The role of CD4 and CXCR4 mediated apoptosis in T cell depletion during HIV-1 infection

Thesis

UNRESTRICTED.

The role of CD4 and CXCR4 mediated apoptosis in T cell depletion during HIV-1 infection

Elena Ritsou

BSc (Hons) BiolSci.

A thesis submitted for the degree of Doctor of Philosophy

German Cancer Research Centre (DKFZ)

Open University

2001

Submission date 6 November 200
AWARD DATE: 19 DECEMBER 2001

DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis is my own, except where stated in the text.

Elena Ritsou

ACKNOWLEDGEMENTS

Many thanks to my immediate supervisor Professor Peter Krammer for giving me the opportunity and all the support I needed to learn and work in his laboratory. Moreover, I am grateful to him for giving me the unique opportunity to develop my singing and acting skills in our unforgetable TITANIC performance!

Many thanks to my secondary supervisor Dr. Sarah E.M Howie who provided me with encouragement and ideas.

I am grateful to everyone in the Krammer lab past and present, for providing a great working environment. Many thanks to Dr. Ingo Shmitz and Andreas Krueger for their help and constructive critisism with biochemical experiments, to Axel Benner for the statistical analysis, to Dr. Ana Martin-Villalba for help with the confocal microscopy, and to Dorothee Koppenhöfer and Dorothee Süss for excellent technical assistance.

Il y a qu'un problème philosophique vraiment sérieux : c'est le suicide
Juger que la vie vaut ou ne vaut pas la peine d'être vécue, c'est répondre à la question fondamentale de la philosophie. Le reste, si le monde a trois dimensions, si l'esprit a neuf ou douze catégories, vient ensuite.

Albert Camus « Le mythe de Sisyphe »

to my parents and my grandmother,

to Manu

CONTENTS

ABSTRACT

AIDS is characterised by a progressive depletion of $CD4^+$ helper T lymphocytes. This process has been shown to involve apoptosis. Several studies have demonstrated that apoptosis can be induced in both infected and non-infected cells by the HIV-1 gp120 surface glycoprotein. Thus, crosslinking of CD4 by gp120 and anti-gp120 antibodies, or specific anti-CD4 antibodies induces apoptosis in uninfected T cells which is partially mediated via sensitization to CD95(Apo-1/Fas) -mediated cell death. It has recently been shown that gp120 induces a novel type of apoptosis in T cells. This was induced via both the CD4 and CXCR4 receptors and was CD95 and caspase independent.

The work described in this thesis investigated the role of CD4 and CXCR4 induced apoptosis as a potential indirect mechanism of T-cell depletion during HIV-1 infection. The relative contribution of the „classical" or „novel" apoptotic pathways engaged by these receptors, upon cell death induction, were investigated in the *in vivo* situation. Data in this thesis demonstrate that peripheral blood lymphocytes (PBLs) from a representative cohort of HIV-1 infected individuals show higher sensitivity to CD4 and CXCR4 mediated apoptosis compared to PBLs from healthy controls. Activation of PBLs from healthy individuals rendered, however, these cells susceptible to CD4 and CXCR4 mediated apoptosis. This supports the hypothesis that the sensitivity of PBLs from HIV-1 infected individuals to CD4 and CXCR4 mediated apoptosis is due to the immune hyperactivation occuring during the course of the infection.

The signaling mechanism of the novel type of cell death was also investigated focusing primarily on CD4 mediated apoptosis. Studies on CD4 mutant receptors showed that the cytoplasmic tail of CD4 is dispensable for the induction of cell death. Preliminary biochemical analysis also failed to detect recruitment of signaling molecules to the CD4 receptor complex. These findings strongly suggest that a molecule adjacent to the CD4 receptor or the CXCR4 receptor may be responsible for the transmission of the apoptotic signal. Studies on the role of mitochondria during CD4 mediated apoptosis showed release of both cytochrome c and AIF. This suggests a

central role for mitochondria in the CD4 induced, caspase independent apoptotic signaling pathway.

Improvement of our understanding of the mechanisms of HIV-1 associated lymphocyte apoptosis, as well as the signaling cascades involved, may lead to therapeutic strategies aimed at intervening with the CD4+ T cell depletion in HIV-1 infected individuals.

I INTRODUCTION

1 Apoptosis

1.1 Definition of apoptosis

Programmed cell death was discovered by C.Vogt in the middle of the 19th century (Vogt, 1842) by the morphology of dying cells during the metamorphosis in amphibians. In more than hundred years after the initial description, programmed cell death was rediscovered many times. The term „apoptosis" was originally used by Kerr et al (1972) to describe the morphological characteristics of a certain type of cell death as opposed to necrosis. During necrosis, the cell swells, its mitochondria dilate, other organelles dissolve and the plasma membrane ruptures releasing cytoplasmic material; this often elicits an inflammatory response.

In contrast, during apoptosis, the cytoplasm shrinks and the chromatin condenses, but the organelles retain their integrity. The plasma membrane blebbs and exposes phosphatidyl serine on its outer surface, which is normally retained in the inner leaflet. However, the plasma membrane does not rupture preventing the release of cellular compounds into the extracellular medium. In vitro, apoptotic cells ultimately fragment into membrane-enclosed vesicles (apoptotic bodies), whereas, *in vivo*, they are recognised and removed by phagocytes, thereby avoiding inappropriate inflammation. Biochemical hallmarks of apoptosis include the activation of endonucleases, DNA degradation into oligonucleosomal fragments and the activation of a family of cysteine proteases called caspases.

Currently, the term apoptosis is used as a synonym of programmed cell death. However, there is accumulating evidence to indicate the existence of genetically programmed cell death pathway(s) that lack certain morphological or biochemical hallmarks of classical apoptosis. This suggests that one needs to take a larger view of

the meaning of the word „apoptosis". The simple original definition of apoptosis needs to be re-evaluated in order to avoid semantic confusion.

1.2 The role of apoptosis in the immune system

Apoptosis is the most common form of death in cells of the immune system. A pivotal process during lymphoid development is the education of immature lymphoid populations to discriminate between self and non-self. In the case of the T cell lineage, immature thymocytes can be positively selected and exit the thymus to populate the peripheral immune system. Alternatively, autoreactive thymocytes are negatively selected and die within the thymus by apoptosis. Similarly, negative selection also takes place in immature B cells in the bone marrow, leading to the elimination of self-reactive B-cell clones.

In the periphery, it is necessary to control proliferation of activated lymphocytes to prevent the accumulation of high levels of cytokines, potentially deleterious to the organism. In the case of T cells, activated cells are induced to undergo apoptosis which prevents the accumulation of toxic levels of cytokines. Increasing evidence suggests that B-cell homeostasis is also maintained by the removal of activated cells by apoptotic mechanisms. In addition to the contribution of apoptosis in the homeostatic control of T and B cell populations, it is also important in the effector function of cytotoxic T cells (CTLs), as these cells kill target cells by the induction of apoptosis in the target. Moreover, autoreactive T lymphocytes which have escaped negative selection in the thymus are eliminated by apoptosis, thus ensuring self-tolerance of the organism.

There are many different pathways via which immune cells can choose to die. In principle, death can be by neglect when the antigen specific receptors of the lymphoid cells are not stimulated or the lymphocytes are deprived of trophic cytokines. In a more active form, death can involve the death-receptor/death-ligand systems (Krammer, 2000).

2 The „death receptors“

Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is defined by similar cysteine-rich extracellular domains (Smith et al., 1994). The death receptors contain in addition a homologous cytoplasmic sequence termed the „death domain“ (Tartaglia et al., 1993; Nagata et al., 1997). Death domains typically enable death receptors to engage the cell`s apoptotic machinery, but in some instances they mediate functions that are distinct from or even counteract apoptosis.

The best characterised death receptors are CD95 (also called Apo-1 or Fas) and TNFR1 (also called p55 or CD120a) (Trauth et al., 1989; Yonehara et al., 1989; Loetscher et al., 1990; Schall et al., 1990). Additional death receptors are Apo-3 (also called DR3, WSL-1, TRAMP or LARD), TRAIL-R1 (DR4), TRAIL-R2 (also called Apo-2, DR5, TRICK2 or KILLER), DR6 and the avian CAR1 (Chinnaiyan et al., 1996; Kitson et al., 1996; Marsters et al., 1996; Pan et al., 1997a; Walczak et al., 1997; Pan et al., 1997b; Sheridan et al., 1997; Screaton et al., 1997a; Schneider et al., 1997; Pan et al., 1998; Brojatsch et al., 1996). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily (Smith et al., 1994). CD95 ligand (CD95L) binds to CD95, TNFα and lymphotoxin α binds to TNFR1, Apo-3 ligand (Apo-3L also called TWEAK) binds to DR3 and TRAIL (also called Apo-2 ligand) binds to TRAIL-R1 and TRAIL-R2 (Chicheportiche et al., 1997; Marsters et al., 1998; Wiley et al., 1995; Pan et al., 1997b; Sheridan et al., 1997).

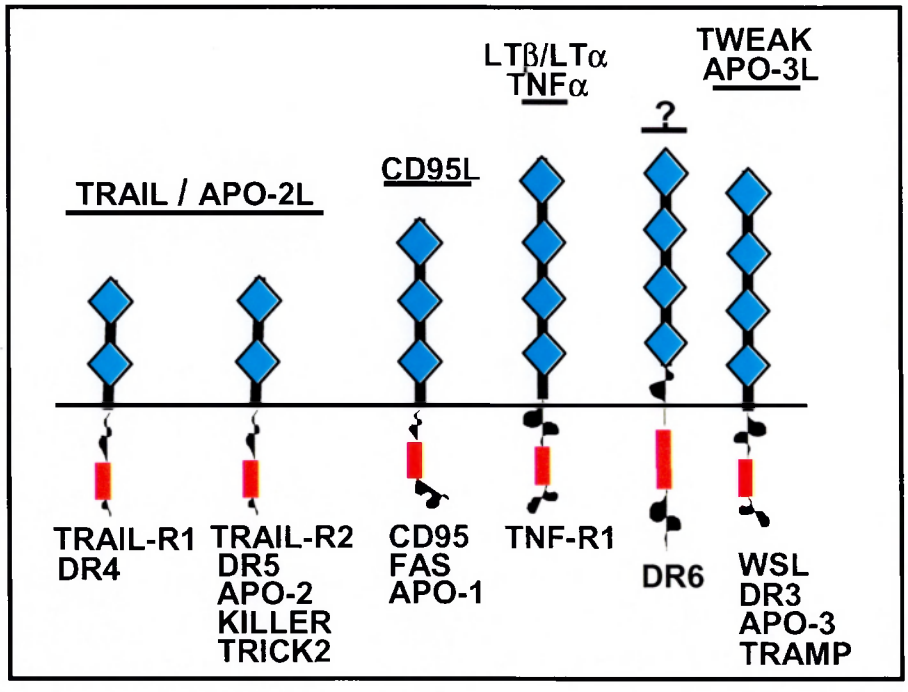

Fig. I.1: Schematic representation of the death receptors and their ligands. The cysteine rich extracellular domains are depicted in blue while the intracellular death domains are depicted in red.

3 The CD95 signal transduction pathway: a paradigm of classical caspase dependent apoptosis

Triggering of CD95 leads to trimerisation of the receptor which is required for transduction of the apoptotic signal. A complex of proteins associates with the activated receptor thus forming the „death inducing signaling complex" (DISC) (Kischkel et al., 1995). The adaptor molecule FADD (CAP1/2, MORT1) binds via its own death domain to the death domain in CD95 (Chinn et al., 1995; Boldin et al., 1995). FADD also carries a death-effector domain (DED) and again by homologous interaction recruits the DED-containing procaspase-8 (also known as FLICE, MACH, Mch5) (Muzio et al., 1996). Next, procaspase-8 is activated proteolytically and active caspase-8 is released into the cytoplasm where it initiates a protease cascade.

3.1 The caspase cascade

Caspase-8 belongs to a growing family of cysteine proteases (Alnemri et al., 1996) which are crucial for the execution of apoptosis. Caspases are synthesized as proenzymes that are activated by proteolytic cleavage.

So far 11 human caspases are known which can be subdivided into three subgroups on the basis of their substrate specificity (Thornberry et al., 1997). Group I includes caspases –1, -4, –5 and –13 which are not implicated in apoptosis but have a role in cytokine processing during inflammation. Group II consists of caspases –2, -3 and –7. The preferred cleavage motif for group II caspases appears in many proteins that are cleaved during cell death, consistent with group II being the major effectors of cell death. Group III include caspases –6, -8, -9 and –10. The cleavage motif for group III caspases is found at the maturation site of most group II and group III caspases. This specificity is consistent with the group III enzymes being upstream activators of the group II effector caspases.

This molecular ordering is exemplified by the caspase-8-mediated activation of caspase-3 and –7 in the CD95 signal transduction pathway (Slee et al., 1999).

Apoptosis can be blocked via caspase-specific inhibitors. Naturally occuring inhibitors include the baculovirus p35 protein (Kamita et al., 1993; Beidler et al., 1995) and the cowpox virus serpin CrmA (Gagliardini et al., 1994; Komiyama et al., 1994). The synthetic tripeptide z-Val-Ala-Asp.fluoromethylketone (zVAD.fmk) and the related compound t-butoxy carbonyl-Asp.fluoromethylketone (BD.fmk) have been described as broad range caspase inhibitors (Sarin et al., 1996; Brown et al., 1998). Due to an aspartate residue mimicking the cleavage site and an fmk group forming a covalent inhibitor/enzyme complex, these inhibitors instantly and irreversibly bind to the catalytic site of caspases (Garcia-Calvo et al., 1998; Thornberry et al., 1997).

Although zVAD.fmk is known to block all so far known caspases (Garcia-Calvo et al., 1998), one cannot exclude that yet unidentified caspases are resistant to this drug. It is not known whether the inhibitor is sufficiently stable inside cells. Moreover, methyl ketone peptide inhibitors have been reported to non-specifically inhibit other cysteine

proteases, such as calpain and cathepsin B (Waterhouse et al., 1998; Schotte et al., 1998).

3.2 The role of mitochondria

In so-called type I cells, the CD95 induced death signal is propagated by activation of large amounts of caspase-8 at the DISC, followed by cleavage of caspase -3 and -7 which in turn cleave vital substrates in the cell. In certain cell types, however, hardly any DISC is formed, so the caspase cascade cannot be propagated directly. These cells (called type II cells) use the mitochondria as amplifiers to initiate the executionary apoptosis caspase cascade (Scaffidi et al., 1998). Activation of mitochondria is mediated by the pro-apoptotic BH3-only Bcl-2 family member Bid. Bid is a substrate of caspase-8 which is activated in low amounts at the DISC (Luo et al., 1998; Li et al., 1998). Truncated Bid translocates to the mitochondria and induces loss of mitochondrial transmembrane potential ($\Delta\Psi m$) and release of apoptogenic factors such as cytochrome c (Kroemer, 1995). Together with the apoptosis protease-activating factor Apaf-1 and procaspase-9 in the cytoplasm, these molecules form a complex called apoptosome (Zou et al., 1997; Li et al., 1998). At the apoptosome pro-caspase-9 is autocatalytically processed to the mature enzyme and initiates a caspase cascade downstream of the mitochondrion (Srinivasula et al., 1998; Zou et al., 1999).

3.3 The death substrates

Activation of caspases does not result in the wholesale degradation of cellular proteins. Rather, caspases selectively cleave a restricted set of target proteins. Several important caspase substrates have been identified in recent years. Molecules involved in DNA repair, such as poly(ADP-ribose) polymerase (PARP) or the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), were described to be cleaved and thereby inactivated by downstream caspase-3 like caspases (Lazebnik et al., 1994; Gu et al., 1995a; Casciola-Rosen et al., 1995; Song et al., 1996b). The ribonucleoproteins U1-70kDa, C1 and C2, components responsible for the splicing reaction of precursor

mRNA are inactivated by cleavage through caspases (Casciola-Rosen et al., 1994; Tewari et al., 1995a; Waterhouse et al., 1996).

Another set of death substrates are structural proteins of the cell. Cleavage of these proteins may account for several of the characteristic morphological features of apoptosis. Thus, cleavage of the nuclear lamins may be required for nuclear shrinking and budding (Rao et al., 1996; Buendia et al., 1999). Loss of overall cell shape is probably caused by the cleavage of cytoskeletal proteins such as actin, fodrin and gelsolin (Mashima et al., 1995; Kothakota et al., 1997).

Moreover, the nuclease that cuts the genomic DNA during apoptosis, to 180 bp fragments, thus leading to DNA ladder formation, is activated by caspase 3. The nuclease (now known as caspase-activated DNase, or CAD or DFF40) pre-exists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD (Nagata et al., 2000). Activation of CAD occurs by means of caspase-3 mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit (Liu et al., 1997; Enari et al., 1998).

4 Caspase independent apoptosis

Since caspases are considered as the main effectors of apoptotic cell demise there is a tendency to conceptually identify apoptosis with its main execution system. It is unlikely that a single execution system is solely responsible for apoptosis. Should this be the case, viruses and transformed cells could have easily escaped or shut down a programme converging on a single execution pathway (Nicotera et al., 2000). Moreover it is difficult to conceive how the plethora of signals causing apoptosis would converge on a single linear pathway. Thus additional interelated or independent pathways may have developed to regulate death.

McCarthy et al. reported that while cells treated with z-VAD.fmk or BD.fmk did not display cleavages of caspase substrates, chromatin condensation or nucleosomal laddering in response to oncogenic and DNA damaging apoptotic stimuli, they

continued to exert other apoptotic features such as cell shrinkage and membrane blebbing and could not be rescued from the death fate. This suggested that certain cytoplasmic hallmarks of apoptosis may be triggered by enzymes other than caspases, but that the nuclear events required caspase activity.

Several studies support the existence of caspase independent apoptotic pathways. These include apoptosis induced via the CD4 and CXCR4 receptors (Berndt et al., 1998), class I MHC antibodies (Woodle et al., 1997), the GD3 ganglioside (De Maria et al., 1997), CD2 and staurosporine (Deas et al., 1998), intracellular acidification (Zanke et al., 1998), CD47 (Pettersen et al., 1999), puromycin (Schlapbach et al., 1997), polyamine analogues (Ha et al., 1998), the retinoid AHPN (Adachi et al., 1998), E4orf4, a novel adenovirus death factor (Lavoie et al., 1998), VP-16, dexamethasone and actinomycin-D (Amarante-Mendes et al., 1998, Brunet et al., 1998, Benson et al., 1998), PML (Quignon et al., 1998) and NO (Okuno et al., 1998). Even some aspects of CD95-induced apoptosis may occur caspase-independently, using the kinase RIP as effector molecule, although in this case necrotic morphologies were observed (Holler et al., 2000). Moreover a nonapoptotic form of programmed cell death, dubbed paraptosis, has been recently described. Although this type of cell death was not inhibited by caspase inhibitors, it was driven by an alternative caspase-9 activity that was Apaf-1 independent (Sperandio et al., 2000).

4.1 Caspase-independent apoptosis effectors

The cellular components involved in caspase-independent apoptosis remain elusive. In most apoptotic systems zVAD.fmk does not block mitochondrial changes such as loss of mitochondrial transmembrane potential, production of oxygen radicals and the release of apoptogenic factors (Green and Reed, 1998; Kroemer, 1998). The recently discovered mitochondrial membrane protein AIF (apoptosis inducing factor) has been proposed as a caspase-independent apoptotic effector (Susin et al., 1999). In addition, Bax and Bax-like proteins, pro-apoptotic homologues of the Bcl-2 family may also be important in this process (Adams and Cory, 2001).

4.1.1 AIF

AIF has been recently identified as a mitochondrial interspace membrane protein with homology to bacterial NADH oxidoreductases. If microinjected into the cytoplasm of normal cells, recombinant AIF causes three hallmarks of apoptosis, namely the dissipation of the mitochondrial transmemrane potential and the release of cytochrome c, the condensation of nuclear chromatin, and the exposure of phosphatidyl serine on the plasma membrane surface. These alterations are rapid and are not prevented by addition of the broad spectrum caspase inhibitor z-VAD.fmk or overexpression of the anti-apoptotic protein Bcl-2 (Susin et al., 1999; Ferri et al., 2000). In cell free systems, addition of recombinant AIF to purified nuclei causes chromatin condensation which is accompanied by large scale DNA fragmentation but not by oligonucleosomal DNA fragmentation.

When cells are induced to undergo apoptosis, AIF is released from mitochondria to the cytosol and to the nucleus. Kinetic studies have shown that the release of AIF coinsides with the dissipation of the mitochondrial transmembrane potential and precedes the release of cytochrome c. Moreover, early caspase independent signs of nuclear apoptosis such as rippled nuclear contours and a partial chromatin condensation seem to involve the translocation of AIF from mitochondria to the nucleus (Daugas et al., 2000).

4.1.2 Bax-like proteins

The Bcl-2 protein family comprises 15 members which play an important role in the regulation of cell survival and apoptosis. These molecules can be subdivided into three groups: the Bcl-2 subfamily which promotes cell survival and the Bax and BH3 subfamilies which facilitate apoptosis (Adams and Cory, 1998). Bax is both a membrane and cytoplasmic protein and there is increasing evidence that the cytoplasmic form undergoes a conformational change and translocates to mitochondrial membranes in response to apoptotic stimuli. In the presence of a caspase inhibitor, overexpression

of Bax-like proteins provoke cell death accompanied by DNA condensation and membrane alterations but no caspase activation or DNA degradation (Xiang et al., 1996; Gross et al., 1998). Bax and Bax-like proteins might mediate caspase independent death via channel-forming activity which could promote the mitochondrial permeability transition or puncture the mitochondrial outer membrane thus leading to release of apoptogenic factors (Green and Reed, 1998).

4.1.3 Non-caspase proteases

Caspase independent apoptotic factors may exert their effect by activating other proteases such as serine proteases, calpains and cathepsins. A recent report has shown that any type of protease, when loaded into cells, can provoke apoptosis like morphology (Williams et al., 1994). Serine proteases have been reported to play a crucial role in the early cleavage of chromatin into 50-300 Kb fragments before they are digested to nucleosome-sized pieces by CAD (Hughes et al., 1998). Moreover, the serine protease granzyme A may mediate caspase-independent CTL/target cell lysis (Chang et al., 1980).

Calpains have been shown to participate in apoptosis in response to several stimuli (Borner and Monney, 1999). Since they are calcium requiring enzymes they may be activated in response to increased cytoplasmic calcium levels, for example due to mitochondrial damage (Berridge et al., 1998). In addition, lysosomal proteases such as cathepsin D have been implicated in INF-γ, CD95 and TNFα induced apoptosis (Deiss et al., 1996).

5 Immunopathology of HIV-1 infection

The type 1 human immunodeficiency virus (HIV-1) is a lentivirus of the family *Retroviridae*. HIV-1 is the causative pathogenic agent of the acquired immune deficiency syndrome (AIDS). Clinically, HIV-1 infection may be divided into three phases: primary infection, asymptomatic period and clinical immunodeficiency.

Initial (acute) infection with HIV-1 results in clinical symptoms within one to three weeks in at least half of those newly infected. These symptoms are similar to influenza infection or mononucleosis, along with a non-pruritic macular erythematosus rash (Fox et al., 1987). During symptomatic primary infection, levels of infectious virus and of infected cells in the circulation are both very high and a substantial reduction in CD4+ T-cell numbers is observed (Daar et al., 1991; McMichael and Phillips, 1997).

Shortly after acute infection, most patients undergo seroconversion. This stage is associated with a dramatic downregulation in the levels of virus replication as determined by plasma viraemia and titers of infectious virus, and an increase in CD4+ T-cell numbers back to normal levels (more than 1000 cells/µl blood) (Pantaleo et al., 1994). This coinsides with the emergence of HIV-specific immune responses. Virus-specific cytolytic T lymphocytes (CTL) appear early and may represent a critical host factor in the control of primary HIV-1 infection (Borrow et al., 1994; Pantaleo et al., 1994). The combined effects of CTL and other elements of the immune response (such as NK cells and humoral immune response) cause the amount of virus in the blood to decrease, however, sterilizing immunity is never achieved.

The next phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency. This period of clinical latency may last from six months to more than fifteen years (Buchbinder et al., 1994). There are two related pathophysiologic characteristics of the asymptomatic phase: ongoing viral replication in the peripheral lymphoid tissues and gradual loss of CD4+ T cells. The rate of decline in CD4+ T cells appears to be determined by the level of ongoing viral replication, as patients with higher plasma virus set points progress to AIDS more rapidly (Mellors et al., 1996).

The final phase of the infection is characterized by the emergence of clinical immunodeficiency. In the year or two before AIDS develops, there is often a more rapid decline in CD4+ T cells. This decline may be preceded by an increase in viral load (Connor et al., 1993). As the CD4 count falls below 200 cells/µl opportunistic infections or cancers begin to occur (Castro et al., 1993). The degree of CD4 decline is an excellent predictor of the risk for particular infections, providing strong support for the notion that the loss of CD4+ T cells is the central cause of immunodeficiency in this disease (Pierson et al., 2000).

6 The role of chemokine receptors in HIV-1 infection

HIV-1 enters target cells by direct fusion of the viral and target membranes. The fusion reaction is mediated by the viral envelope glycoprotein (Env), which binds with high affinity to CD4, the primary receptor on the target cell surface. The notion that a coreceptor is required for HIV-1 entry stemmed from the awareness that CD4 expression is not sufficient to explain HIV-1 tropism for different target cells *in vitro* (Berger et al., 1997). This led to the identification of the CXCR4 and CCR5 chemokine receptors, members of the G protein-coupled receptor superfamily as the principal coreceptors for HIV-1 entry (Feng et al., 1996; Raport et al., 1996).

The type of chemokine receptor which the virus is able to use depends on the sequence of its gp120. The V3 loop of gp120 is particularly important in determining coreceptor usage (Fouchier et al., 1992). The viral DNA encoding this region is highly variable and also mutates during the course of infection thus allowing the virus to change its coreceptor usage and, therefore, phenotype. However, conserved regions of gp120 have also been shown to be important in binding to chemokine receptors (Verrier et al., 1997).

Macrophage-(M)-tropic viruses, which represent the predominant virus population early after infection, use the CCR5 receptor to enter primary CD4+ T cells

and macrophages (R5 viruses). Lab adapted strains grown for many passages on T cell lines use CXCR4 as a coreceptor. Although CXCR4 is present on both macrophages and T cells, most T-cell line adapted viruses can only use CXCR4 on primary T cells or T cell lines, the CXCR4 coreceptor on macrophages appears to be unavailable for binding in this case (Yi et al., 1999). Dual tropic viral strains which can use both CCR5 and CXCR4 are able to infect both macrophages and T cells. CXCR4 using (X4) viruses and/or viruses with dual tropism (R5/X4) typically emerge later in disease in temporal association with rapid CD4+ T cell decline and progression to AIDS (Connor et al., 1997; Goudsmit et al., 1995; Xiao et al., 1998).

Additional complexity results from the findings that HIV-1 coreceptor activity is not limited to CXCR4 and CCR5. Coreceptor activity has been also demonstrated for several other chemokine receptors such as CCR1, CCR2b and CCR3 (Choe et al., 1996; Doranz et al., 1996).

The realization that CCR5 is the molecular factor mediating entry of the preferentially trasmitted M-tropic HIV-1 variants led to a focus on this receptor as a possible determinant of transmission. Evidence came from the discovery of a mutant CCR5 allele designated CCR5 Δ32 and the association of this allele with resistance to HIV-1 infection. CCR5 Δ32 has a 32 base pair deletion leading to a truncated protein product which is not expressed on the cell surface (Liu et al., 1996; Martinson et al., 1997). The mutant allele is common in Caucasians, heterozygotes and homozygotes represent ~ 20% and ~1% of this population, respectively. Homozygocity for Δ32 does not confer a selective disadvantage on healthy individuals, suggesting that normal CCR5 function is dispensable. It confers however a protective effect on transmission of HIV-1 (Connor et al., 1996; Rana et al., 1997). Rare HIV-1 infected Δ32/Δ32 individuals have been reported, thus demonstrating that CCR5 is not absolutely required for HIV-1 transmission and raising the speculation that CXCR4 was the coreceptor responsible for initiating infection (Biti et al., 1997; O`Brien et al., 1997; Theodorou et al., 1997).

Chemokine coreceptor and chemokine genetic polymorphisms have also been correlated with HIV-1 disease progression. Thus, Δ32 heterozygocity has been

correlated with delayed progression to AIDS and shows a higher genotypic frequency in cohorts of long term nonprogressors (Dean et al., 1996). In addition, another CCR5 gene polymorphism, CCR5 59029 G/A, as well as a CCR2 gene polymorphism, CCR2-64I, correlate with slower disease progression (McDermott et al., 1998; Smith et al., 1997). Like CCR5 Δ32, CCR2-64I is enriched among long-term non progressors and reduced in rapid progressors (Smith et al., 1997).

A genetic polymorphism, designated SDF-1 3'A, has also been identified in one of the two known chemokine ligand for CXCR4, SDF-1β. SDF-1 3'A is found in all racial groups tested and homozygote individuals represent ~ 5% of Caucasians. A strong association has been described between the homozygous 3'A/3'A genotype and delayed onset of AIDS while the effect was reported to be increased in later stages of HIV-1 infection (Winkler et al., 1998).

7 The role of apoptosis in the pathogenesis of HIV-1 infection

7.1 Apoptosis as the basis of CD4+ T-cell depletion during HIV-1 infection

Although CD4+ T-cell production is impaired in HIV-1 infected patients (Hellerstein et al., 1999), there is currently overwhelming evidence that the primary basis of T-cell depletion is increased apoptosis of CD4+ and CD8+ T cells (Gougeon et al., 1996; Badley et al., 2000). Because HIV-1 is cytopathic for T cells *in vitro*, it was originally presumed that direct viral cytopathic effects were responsible for T cell depletion (Heeney, 1995). However, subsequent studies have shown that only a minor fraction of lymphocytes (<0.01%) is physically infected by HIV-1 (Chun et al., 1997; Simmonds et al., 1990). Moreover, HIV-1 infected individuals experience a loss of CD8+ T-cells, NK cells and neurons although these cells are not infected by HIV-1 (Gougeon et al., 1996; Oyaizu and Pahwa, 1995). Therefore, cytopathic effects of the virus due to direct infection cannot solely account for the enhanced apoptosis of

lymphocytes seen in infected persons. This indicated that other indirect mechanism(s) are involved.

7.1.1 Indirect mechanisms of HIV-associated apoptosis

Finkel et al. showed in HIV-1 infected children and simian immunodeficiency virus-infected macaques that predominantly non-infected cells are eliminated by apoptosis. In addition, infected and non-infected T cells from HIV-1 infected individuals show enhanced spontaneous apoptosis *in vitro* and are more sensitive to activation-induced cell death (AICD) than T cells from non-infected subjects (Groux et al., 1992; Meyaard et al., 1992; Gougeon et al., 1993). Enhanced sensitisation is paralleled by enhanced expression of CD95 and its ligand (CD95L) and by enhanced sensitivity to CD95 mediated apoptosis (Debatin et al., 1994; Baumler et al., 1996). Moreover, T cells from HIV-1 infected individuals show susceptibility to TRAIL mediated apoptosis in contrast to T cells from healthy control subjects (Jeremias et al., 1998). The TRAIL system may also contribute to the enhanced T-cell AICD observed in HIV-1 positive individuals (Katsikis et al., 1997). A recent study, using an HIV-1 infection mouse model, also suggests that large numbers of uninfected CD4+ T cells undergo TRAIL-mediated apoptosis (Miura et al., 2001).

7.1.2 Apoptosis induced by HIV-1 encoded proteins

Although the aberrant immune activation occuring during HIV-1 infection can lead to lymphocyte apoptosis, considerable data indicate that there are additional distinct mechanisms by which HIV-1 induces apoptosis (Badley et al., 2000). Indeed several HIV-1 encoded proteins such as Tat, Nef and Vpr have been implicated in enhancement or direct induction of apoptosis. Tat may induce apoptosis in uninfected T cells by altering the redox state and by CD95 dependent mechanisms (Westendorp et al., 1995). Recently; Tat has been also shown to upregulate TRAIL in macrophages, which could then induce apoptosis of bystander T cells (Zhang et al., 2001). Nef has also been suggested as a potential mediator of CD95-independent apoptosis (Okada et al., 1997). In addition, Vpr has been shown to induce apoptosis via a direct effect on mitochondrial permeability (Jacotot et al., 2000) and via induction of cell cycle arrest (Stewart et al., 1997).

7.1.2.1 Gp120-induced apoptosis

The HIV-1 envelope glycoprotein gp120, which binds and crosslinks the CD4 receptor and the chemokine coreceptors, has also been described as a potent inducer of apoptosis (Laurent-Crawford et al., 1993; Corbeil et al., 1995; Ohnimus et al., 1997). HIV-1 envelope interaction with CD4 can induce apoptosis of HIV-1 infected T cells via caspase activation but independently of CD95 and Bcl-2 (Gandhi et al., 1998; Kolesnitchenko et al., 1997). Crosslinking of CD4 by gp120 and anti-gp120 antibodies, or specific anti-CD4 antibodies, also induces apoptosis in uninfected T cells. This is partially mediated via sensitization to CD95-mediated cell death (Banda et al., 1992; Westendorp et al., 1995).

The recent discovery of the chemokine coreceptor CXCR4 prompted the investigation of its role in HIV-1 envelope induced apoptosis. Indeed several reports have implicated CXCR4 in gp120 induced apoptosis. Thus, Hesselgesser et al. reported that gp120 induced neuronal apoptosis is mediated by CXCR4. Cell surface expressed HIV-1 envelope also triggered apoptosis in CXCR4 transfected HEK-293 cells, in a caspase dependent manner (Biard-Piechaczyk et al., 2000). Blanco et al. reported that HIV-1 envelope induced apoptosis in syncytium-forming and in single uninfected lymphocytes was blocked by a CXCR4 antagonist. In addition, infection of lymphocytes and lymphoid tissue with CXCR4 dependent HIV-1 led to depletion of CD4 positive T cells thus suggesting a critical pathogenic role for gp120-CXCR4 interactions (Penn et al., 1999).

Recent studies have shown that stimulation of CD4 and CXCR4 via gp120 crosslinked by anti-gp120 antibodies, or via specific anti-CD4 and anti-CXCR4 antibodies, induces a novel type of apoptosis in T cells (Berndt et al., 1998). This new type of cell death showed rapid kinetics, was independent of caspases and did not involve the CD95-, TNF- and TRAIL-death systems. Although oligonucleosomal DNA degradation (a hallmark of classical apoptosis) was not observed several characteristic features of apoptotic cell death were detected, such as cytoplasmic and chromatin condensation, integrity of plasma membrane and exposure of phosphatidyl serine as well as dissipation of the transmembrane mitochondrial potential (figure I.2). Interestingly, apoptosis was observed exclusively in CD4+ but not in CD8+ T cells thus

suggesting a significant role for this phenomenon in T helper cell depletion during HIV-1 infection.

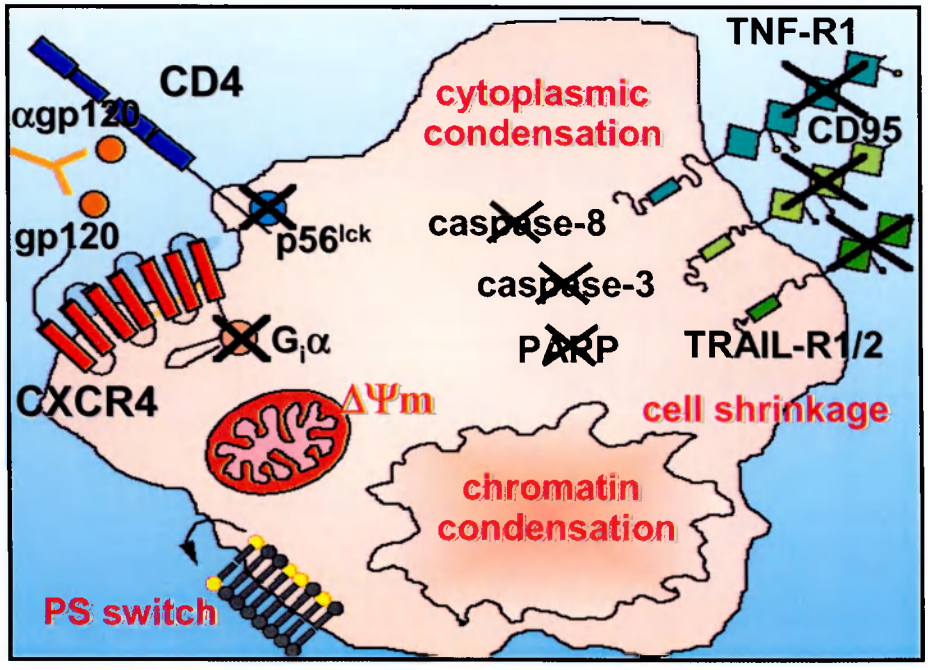

Fig. I.2: Schematic representation of the characteristics of the novel type of CD4 and CXCR4 mediated apoptosis. Triggering of the CD4 and CXCR4 receptors by recombinant gp120 crosslinked via anti-gp120 antibodies leads to a novel type of cell death. This involves condensation of the cytoplasm and chromatin, reduction of the mitochondrial transmembrane potential and exposure of phospatidyl serine on the plasma membrane (PS translocation). The death receptors CD95, TNF-R1, TRAIL-R1 and TRAIL-R2 and components of the classical apoptotic signaling pathway , such as caspases and caspase substrates (PARP); do not play a role in this type of apoptosis. The death signal is not mediated by the CD4 associated tyrosine kinase $p56^{lck}$ or the CXCR4 associated GTPase $G_i\alpha$.

8 Signal transduction via the CD4 and CXCR4 receptors

8.1 CD4 signal transduction

Productive antigen receptor signaling in helper T cells requires coengagement of the CD4 coreceptor. CD4 binding to MHC class II molecules results in the stabilization of the TCR-MHC II interaction and activation of a signaling pathway initiated by the CD4-associated protein tyrosine kinase (PTK) Lck (Weiss and Littman, 1994). CD4 engagement independently of the TCR has been shown to trigger the phosphorylation of a subset of the phosphoproteins triggered by the TCR, supporting the view that TCR and CD4 share a wide array of common signaling mediators. Thus CD4 triggering leads to phosphorylation of the PTKs Lck and Fyn, the adaptor molecule Shc, the MAP kinase Erk 2 and PI-3 kinase (Ulivieri et al., 1999). Coengagement of CD4 during TCR signaling is also necessary for the phosphorylation of the PTK ZAP-70 and the adaptor molecule c-Cbl (Murphy et al., 1998). Moreover CD4 coengagement has been shown to promote the phosphorylation of the membrane-associated adaptor LAT by ZAP-70, as LAT associates directly with CD4 and competes with Lck for the binding site (Bosselut et al., 1999).

Binding of HIV-1 envelope glycoprotein to CD4 has been shown to activate the MEK/ERK kinase pathway resulting in the expression of inflammatory genes (Popik et al., 1998). However whether CD4 signaling mediates HIV-induced apoptosis remains conroversial. Moutouh et al reported that initial signaling through CD4 was critical for HIV-1 induced apoptosis although this was independent of $p56^{lck}$. Other studies have indicated that signaling via CD4 may be dispensable for apoptosis induction (Guillerm et al., 1998; Jacotot et al., 1997). Moreover the recently described CD4 mediated novel type of apoptosis was also independent of $p56^{lck}$ signaling.

8.2 CXCR4 signal transduction

Signal transduction by chemokine receptors leads to the activation of G proteins and phospholipase C and the elevation of cytosolic free calcium (Baggiolini et al., 1997). CXCR4-mediated responses are pertussis toxin (PTX) sensitive, indicating a role for $G_i\alpha$ in this signaling. Indeed, $G_i\alpha$ was shown to rapidly associate with CXCR4 after SDF-1α binding followed by receptor tyrosine phosphorylation via the JANUS kinase family members JAK2 and JAK3. Moreover SDF-1α induced the activation and association of the tyrosine phosphatase Shp1 with CXCR4 in a $G_i\alpha$-dependent manner (Vila-Coro et al., 1999). Stimulation of CXCR4 by SDF-1 also results in increased phosphorylation of focal adhesion components, including the tyrosine kinase RAFTK (Pyk2), Crk and paxillin, activation of MAP kinases (Erk1 and 2) and PI3 kinase (Ganju et al., 1998).

HIV-1 envelope glycoprotein binding to CXCR4 also leads to the activation of signal transduction pathways. Stimulation of CXCR4 by gp120 induced Pyk2 phosphorylation which was inhibitable by PTX, thus implicating G_i-linked pathways in this response (Davis et al., 1997). However, viral entry does not depend on G_i-coupled signaling and mutations that ablate signal transduction do not affect coreceptor activity (Cocchi et al., 1996; Farzan et al., 1997).

HIV-1 binding to CXCR4 in neuronal cells induced G_i-protein signaling which correlated with caspase activation and apoptosis (Zheng et al., 1999). However, CXCR4 induced apoptosis in T lymphocytes was found to be independent of G_i-protein signaling and caspases (Berndt et al., 1998; Blanco et al., 1999) and did not seem to involve phosphorylation of the cytoplasmic tail (Biard-Piechaczyk et al. 2000).

9 Aims of project

Improvement of our understanding of the mechanisms of HIV-1 associated lymphocyte apoptosis, as well as the signaling cascades involved, may lead to therapeutic strategies aimed at intervening with the CD4+ T cell depletion in HIV-1 infected individuals.

The work described in this thesis was undertaken in order to investigate the hypothesis that CD4 and CXCR4 mediated apoptosis may be a potential indirect mechanism of T-cell depletion during HIV-1 infection, thus contributing to HIV-1 disease pathogenesis.

The relative contribution of the „classical" or „novel" apoptotic pathways engaged by these receptors, upon cell death induction, was investigated in the *in vivo* situation. Furthermore, factors determining sensitivity versus resistance to CD4 and CXCR4 induced apoptosis were examined.

The work also aimed to investigate some of the signaling events involved in the „novel" type of apoptosis triggered by the CD4 and CXCR4 receptors. For this purpose the work was particularly focused on the CD4 triggered apoptotic cascade.
Specific aims of the work were:

- **CHAPTER 1:** To investigate the sensitivity of PBLs from HIV-1 infected individuals to CD4 and CXCR4 mediated apoptosis in comparison to PBLs from healthy controls.
- **CHAPTER 2:** To investigate the apoptotic pathway engaged by the CD4 and CXCR4 receptors in PBLs from HIV-1 infected individuals.
- **CHAPTER 3:** To investigate whether PBLs from healthy individuals can be sensitized to undergo CD4 and CXCR4 mediated apoptosis via activation.
- **CHAPTER 4:** To gain some insight into the structural requirements of the CXCR4 and CD4 receptor for the propagation of the apoptotic signal.
- **CHAPTER 5:** To investigate the role of phosphorylation and recruitment of signaling molecules to the CD4 receptor complex during apoptosis.
- **CHAPTER 6:** To investigate the role of AIF and mitochondria during CD4 mediated apoptosis.

II Materials and Methods

1 Materials

1.1 Chemicals

All chemicals were, unless otherwise stated, purchased from the following companies: Serva (Heidelberg, Germany), Fluka (Neu-Ulm, Germany), Sigma (Munich, Germany), Roth (Karlsruhe, Germany) and Merck (Darmstadt. Germany). Radioactive reagents were purchased from Amersham-Buchler (Braunschweig, Germany).

1.2 Buffers

DNA Sample Buffer (10 x):	50 % (v/v)	Glycerol
	0.42 % (w/v)	Bromophenol blue
	0.42 % (w/v)	Xylene cyanole
Nicoletti Buffer:	0.1 % (w/v)	Sodium citrate
	0.1 % (w/v)	Triton X-100
	50 µg/ml	Propidium iodide
Cell Lysis Buffer		
(Eukaryotic cells):	150 mM	NaCl
	30 mM Tris-HCl, pH = 7.5	
	1 mM	PMSF
	10 % (w/v)	Glycerol
	1 % (w/v)	Triton X-100
	0.5 µg/ml	Antipain
	0.5 µg/ml	Chymostatin A
	0.5 µg/ml	Leupeptin
	0.5 µg/ml	Pepstatin

Additionally, for phosphorylation studies the following were added :

	10 mM	NaF	
	1 mM	Na-orthovanadate	
PBS:	137 mM	NaCl	
	8.1 mM	Na_2HPO_4	
	2.7 mM	KCl	
	1.5 mM	KH_2PO_4	pH = 7.4
TBE (10 x):	0.45 M	Tris	
	0.45 M	Boric acid	
	10 mM	EDTA	pH = 8.3
TE:	10 mM	Tris	
	1 mM	EDTA	pH = 7.5
Reducing sample-buffer (5x):	50 % (v/v)	Glycerol	
	10 % (w/v)	SDS	
	50 mM	Tris, pH = 6.8	
	25 % (v/v)	β-Mercaptoethanol	
	0.25 mg/ml	Bromophenol blue	
Stacking gel (5 %):	24 mM	Tris-HCl, pH = 6.8	
	5 % (w/v)	Acrylamide	
	0.1 % (w/v)	SDS	
	0.1 % (w/v)	APS	
	0.1 % (w/v)	TEMED	
Running gel:	37.5 mM	Tris-HCl, pH = 8.8	
	7.5-15 %(w/v)	Acrylamide	
	0.1 % (w/v)	SDS	
	0.03 % (w/v)	APS	

	0.1 % (w/v)	TEMED
Electrophoresis buffer	25 mM	Tris-Base
(SDS-PAGE):	0.19 M	Glycine
	1 % (w/v)	SDS
Fixing solution:	20 % (v/v)	Methanol
	10 % (v/v)	Acetic acid
Transfer buffer:	25 mM	Tris
(Western Blot)	0,19 M	Glycine
	20 % (v/v)	Methanol
	0.037 % (w/v)	SDS

Nitrocellulose Wash Buffer: PBS with 0.1 % Tween-20
(TPBS)

1.3 Biological Material

1.3.1 Bacterial strains

E.coli TOP 10F´, competent cells	Invitrogen
E.coli XL1 blue	Stratagene

1.3.2 Cell lines

Cell line	Origin
BJAB	EBV-negative human Burkitt-like lymphoblastoid line
H9	human T-cell-leukemia line
HPBALL	human T-cell-leukemia line
JURKAT	human T cell leukemia line
L929	murine fibrosarcoma line

1.4 Media

1.4.1 Cell culture media

RPMI 1640	Gibco BRL, Eggenstein
Iscove's Modified Dulbecco's Medium	Gibco BRL, Eggenstein

The media were supplemented with the following additives prior to use:

10% (v/v)	FCS	Gibco BRL, Eggenstein
10 mM	HEPES	Gibco BRL, Eggenstein
50 µg/ml	Gentamycin	Gibco BRL, Eggenstein

The following antibiotic was used for the selection of transfected eukaryotic cells:

Geneticin (G418)	Sigma, Deisenhofen

For cell labelling the following "deficient" medium was used:

[^{35}S]-Methionine/Cysteine Cell Labelling: RPMI defficient medium (Gibco BRL, Eggenstein,Germany Cat.no.: 51871-010), supplemented with 10% dialysed FCS and all missing amino acids except cysteine and methionine.

1.4.2 Bacterial cell culture media

LB-Medium:	10 g/l	Casein Hydrolysate
	5 g/l	Yeast Extract
	10 g/l	NaCl
	pH 7.2	adjusted with 1 N NaOH

LB-Agar:	1.5%	Bacto-Agar	in LB-Medium
LB-Agar selective plates:	1.5%	Bacto-Agar	in LB-Medium
	Ampicillin 50 -100 µg/ml		
	or	Kanamycin 25 µg/ml	

1.5 Antibodies

1.5.1 Polyclonal Antibodies

Name	Antigen	Source, Reference
AIF rabbit anti-serum	Apoptosis Inducing Factor	Susin et al., 1999
Lck (2102) rabbit IgG	p56 lck	Santa Cruz Biotechnology, Heidelberg, Germany
Goat anti-CD4	Human CD4	R & D systems
Sheep anti-Mouse IgG	Mouse IgG	Boehringer,Mannheim, Germany
Goat anti-Mouse IgG(Fab')$_2$ ß-Phycoerythrin conjugated	Mouse IgG	Dianova, Germany
Goat anti-IgG1, HRPO	Mouse IgG1	Southern Biotechnology, Birmingham, USA
Goat anti-IgG2a, HRPO	Mouse IgG2a	Southern Biotechnology, Birmingham, USA
Goat anti-IgG2b, HRPO	Mouse IgG2b	Southern Biotechnology, Birmingham, USA
Goat anti-Rabbit , HRPO	Rabbit IgG	Santa Cruz Biotechnology, Heidelberg, Germany

1.5.2 Monoclonal Antibodies

Name	Antigen	Source, Reference
12G5 (IgG2a)	CXCR4	Pharmingen, San Diego, USA
12G5 (IgG2a)	CXCR4	R & D systems
3G8 (IgG1)	CD16	Immunotech
HP2/6 (IgG2b)	CD4	Carrera et.al., 1987
Leu3a (IgG1)	CD4	Becton Dickinson
FITC-conjugated anti-CD4 RPA-T4 (IgG1)	CD4	Pharmingen, San Diego, USA
10H2	CXCR2	Genetech, CA,USA
anti-APO-1 (IgG3 and IgG1)	CD95	Trauth et al., 1989
FITC-conjugated mouse IgG1		Pharmingen, San Diego, USA
C15 (IgG2b)	Caspase-8 p18 subunit	Scaffidi et al., 1997
anti-FADD (IgG1)	FADD	Transduction Laboratories, Lexington, USA

CII10 (IgG1)	PARP	Grube and Bürkle, 1992
anti-Cytochrome *c* (IgG2b) (AS 93-104)	Cytochrome *c*	Pharmingen, San Diego, USA
anti-COX4 (IgG2a)	Cytochrome oxidase subunit IV	Molecular Probes, Oregon, USA
M-A251 (IgG1)	CD25	Pharmingen, San Diego, USA
FN50 (IgG1)	CD69	Pharmingen, San Diego, USA
HD37 (IgG2a)	CD19	Pezzutto et al. 1987
OKT4 (IgG2b)	CD4	Ortho-Clinical Diagnostics,Germany
OKT8 (IgG2a)	CD8	Ortho-Clinical Diagnostics,Germany
UCHL1 (IgG2a)	CD45RO	Pharmingen, San Diego, USA
HI100 (IgG2b)	CD45RA	Pharmingen, San Diego, USA
H4149	Hsp60	Sigma-Aldrich, Germany
PY99 (IgG2b)	Phospho-Tyrosine	Santa Cruz Biotechnology, Heidelberg, Germany

1.6 Molecular Biology Materials

1.6.1 Vectors

Name	Source
pcDNA3	Invitrogen
pBluescript II SK+	Stratagene
pEGFP-N1	Clontech

1.6.2 Enzymes

Enzyme	Source
Calf Intestinal Alkaline Phosphatase (CIAP)	New England Biolabs, Schwalbach
Pfu DNA Polymerase	Promega, Mannheim
T4 Polynukleotide kinase	MBI Fermentas
T4 DNA Ligase	MBI Fermentas

All restriction endonucleases used were purchased from Boehringer Mannheim and MBI Fermentas.

1.6.3 Primers (Oligonucleotides)

Name	Nucleotide Sequence (5′-3′)
CXCR4 sense	CATTCCCGCGGATGGAGGGGATC AGTATATAC
CXCR4 antisense	TGCTCTAGAGCTGGAGTGAAAAC TTGAAG
CXCR4 Δcyto sense	ATACAAGCTTATGGAGGGGATC AGTATATACACTTC
CXCR4 Δcyto antisense	ATACTCTAGATTAGAGTGCGTGC TGGGCAGAGGTTTTAA
CXCR2 sense	CATTCCCGCGGATGGAGAGTGAC AGCTTTGAAG
CXCR2 antisense	TGCTCTAGAGAGAGTAGTGGAAG TGTGCCC
CXCR4 1 sense	CCCAAGCTTATGGAGGGGATCAG TATATAC
CXCR4 425 antisense	GTGGCGTGGACGATGGCCAG
CXCR2 432 sense	TTACCTGGCCATTGTCCATG
CXCR2 1080 antisense	CCGGAATTCGAGAGTGGAAGTG AGCCC
CXCR2 1 sense	CCCAAGCTTATGGAAGATTTTAA CATG
CXCR2 90 antisense	GGGGACGGTAGTGCTGTAAC
CXCR4 82 sense	GAAGAAAATGCTAATTTC
CXCR4 729 antisense	GATGAGTGTGGTCAAGTTGAG
CXCR2 739 sense	CACCGGGCCATGCGGGTC

1.7 Kits

BCA Protein Assay Kit	Pierce
Chemiluminescence Kit (ECL)	Amersham-Buchler
Qiagen Plasmid Maxi Kit	Qiagen
Qiagen Gel-Extraction Kit	Qiagen
ApoAlert Cell Fractionation Kit	Clontech

1.8 Cytokine Receptor Constructs

CD95-R-Fc construct	Dhein et.al, 1995
TNF-R2-Fc construct	Peppel et.al., 1991
TRAIL-R2-Fc construct	Walczak et al., 1997

The Receptor-Fc constructs are chimaeras composed of a mouse IgG1 Fc portion and two extracellular domains from the respective receptors, which replace the immunoglobulin antigen binding domains.

1.9 Molecular weight markers

1.9.1 Protein Markers

SeeBlue™ Plus2 Pre-Stained Standards	Novex
Rainbow Marker	AP-Biotech

1.9.2 DNA Markers

Ladder Mix (from 100 bp to 10kb)	MBI Fermentas
MidRange PFG Marker I (Pulse Field Gel Electrophoresis)	New England Biolabs

1.10 Apparatus

Apparatus		Source
Autoradiography cassette		Suprema
Blotting apparatus Semi-Dry	20 x 25 cm	CTI
Centrifuges:	Biofuge A	Heraeus
	Omnifuge 2.ORS	Heraeus
	Centrifuge 5402	Eppendorf
	Sorvall RC 3B PLUS, 5C PLUS	DuPont
Stericult Incubator		Forma Scientific
Electrophoresis Power Supply Consort 865		Renner
Electroporation apparatus	Gene Pulser II	Biorad
Film developing apparatus Curix 160		Agfa-Gevaert

Flow cytometer FACScan		Becton Dickinson
Gel elecrophoresis chambers:	Horizon 11.14	Gibco BRL
	SDS-PAGE	CTI
	Bio-RadCHEF-DR II (nucleic acid PAGE)	Biorad
	Minigel chamber	Biorad
Microscopes:	Light microscope ID 02	Zeiss
	Phase contrast microscope Labovert FS	Leitz
	Confocal microscope	Olympus
PCR maschine	Gene Amp 9700	Perkin Elmer
Photo unit	UV-Flächenstrahler	Konrad Benda
	Kamera RA1	Kaiser
	Video Graphic Printer UP-860 CE	Sony
Photometer U-1100		Hitachi

2 Methods

2.1 Cell culture methods

2.1.1 Eukaryotic cell culture

All cell culture work was performed under sterile conditions in order to avoid contamination with bacteria, yeast or fungi. The cell culture material used was gamma-irradiated and all solutions and media were sterile filtered.
Suspension cells were kept in a minimum density of $1x10^5$ cells/ml and a maximum of $1x10^6$ cells/ml. Various cell types were cultured in the following media:
RPMI 1640 medium: BJAB, HPBALL, H9, primary human lymphocytes
Iscove's Modified Dulbecco's Medium:Jurkat

BJAB tranfected cells were cultured in RPMI 1640 medium containing 4 mg/ml Geneticin.

2.1.2 Cell counts determination

Cell counts were performed by using a Neubauer cell count chamber and Trypan blue dye exclusion. A fresh cell culture aliquot was diluted 1:1 with 0,1% Trypan blue solution in order to discriminate between live and dead cells. Trypan blue binds to cytoplasmic protein structures but can only enter cells with leaky plasma membranes. These cells represent the dead cells. The trypan blue negative cells were counted in 2 big quadrats of the Neubauer chamber and the cell count (live cells /ml culture) was determined according to the following formula:

Count 2x16 squares=N

(N x dilution factor) / (2 x 100)= ....$x10^6$ live cells/ml culture

2.1.3 Freezing and thawing cells

Freezing

$5x10^6$ to $1x10^7$ cells were centrifuged (10 min, 1200 rpm, 4°C) and the pellet was resuspended in 1 ml chilled freezing medium (90%FCS, 10% DMSO). The cells were quickly frozen at –80°C and kept for 48-72 h before being transferred to a liquid Nitrogen tank.

Due to its high Dielectric constant Dimethyl-sulfoxide (DMSO) prevents the formation of destructive ice crystals at low temperatures while above and in the range of the freezing point it allows solubilisation of cell membranes. In order to minimize the toxic effects of DMSO, the freezing solution was kept cold during the procedure which was carried out in the fastest time frame possible.

Thawing

Liquid nitrogen frozen cells were defrosted in a 37°C waterbath. In order to ensure reaching a temperature close to the freezing point, which protects the cells from the toxic effects of RT freezing medium, resting ice particles should be seen in the cell solution. The cells were then tranferred to a tube containing 10 ml of warm medium and centrifuged at 1500 rpm for 5 min. The washing step was repeated in order to ensure complete removal of the freezing medium. The cells were then transferred to a 25 cm^2 cell culture flask at a density of $5x10^5$ cells/ml and kept in an incubator at 37°C, 95% humidity and 5% CO_2 content.

2.2 Immunological Methods

2.2.1 Human Peripheral Blood Lymphocyte Purification

Human peripheral blood lymphocytes (PBLs) were isolated by Ficoll-Hypaque density centrifugation. Briefly, blood was obtained from adult volunteers by intravenous puncture into heparinised syringes (5000 units Heparin sodium salt/ml of blood). The blood was diluted 1:1 with 37°C RPMI medium, slowly layered onto 37°C Ficoll (Biochrom, Berlin) and centrifuged (2420 rpm, 20 min, 20°C). The mononuclear cell

layer was gently aspirated and washed twice with 3 volumes PBS at 1200 rpm for 10 min at 20°C (Omnifuge 2.ORS). The cells were then resuspended in complete RPMI medium. Monocytes and macrophages were depleted by adherence to cell culture flasks for 2 h at 37°C. The non-adherent lymphocytes were recovered and counted (see II.2.1.2). PBL numbers typically recovered using this method ranged from 1-2x10^6 cells /ml of blood.

2.2.2 Isolation of PBL subpopulations

PBL subpopulations were isolated using an immunomagnetic cell separation method. This method is based on antibody mediated coupling of cells to "magnetic beads": uniform, superparamagnetic monodisperse particles with identical physical and chemical composition. The magnetic beads used were coupled to anti-Mouse-IgG and therefore bound to cells bearing mouse antibodies against surface markers.

Briefly, 5x10^7 PBL were centrifuged (1200 rpm, 10 min) and resuspended in 400 µl culture medium. 25 µg/ml of a monoclonal mouse antibody against the antigen of interest (i.e CD4, CD8, CD45RA, CD45RO) were added and the cells were incubated on a rotator for 20 min at 4°C. The cells were washed once to remove unbound antibody and the cell pellet was resuspended in 400 µl beads-suspension (8x10^7 magnetic beads). The cells were further incubated as before and then transferred to a magnetic apparatus for 2 min at RT. The magnetic beads with the bound cells were directed towards the apparatus while the "negatively selected" unbound cells remained in suspension. The cells were then gently recovered into a new tube washed and counted. This method of purification typically generated ≥ 90% pure populations.

The "negative selection" of PBL subpopulations here described avoids unwanted activation of the cell population of interest via antibody binding.

2.2.3 Immunofluorescence staining

2.2.3.1 Immunofluorescence staining of surface molecules

Surface antigen expression analysis was performed by using 3-5x10^5 cells of interest . The cells were centrifuged (5 min, 1500 rpm) and the pellet was resuspended in 100 µl PBS. 1µg of relevant monoclonal primary antibody was added and incubated for 15-60 min at 4°C in order to prevent internalisation of the surface molecules. Unbound antibody was washed away by adding 2 ml of medium and centrifugation (10 min, 1200 rpm). The cells were then resuspended in 50 µl medium containing 10µg/ml fruorescent dye-conjugated secondary antibody and incubated at 4°C for 15 min. The cells were washed twice with PBS and resuspended in 200 µl fixing solution (PBS/1% formaldehyde). Thus the stained samples could be kept for several weeks in the dark, at 4°C.

Immunofluorescent cells were analysed in a FACScan flow cytometer (Becton Dickinson, CA, USA). The cells were taken up via a capillary and excited by an Argon laser with a wavelength of λ=488 nm. The wavelength of the light emitted by the excited electrons was dependent on the fluorescent dye used. The light was then collected and redirected to detectors via different mirrors and filters. These detectors provided information concerning the size ("forward scatter", FSC), granularity ("side scatter", SSC) and fluorescence ("fluorescence detector", FL) of the cells analysed.

2.2.3.2 Intracellular Immunofluorescence staining

In order to analyse the expression of intracellular proteins the cells were fixed and permeabilised prior to the addition of the antibodies. Briefly, 5x10^6 cells of interest were washed once with PBS (1200 rpm, 5 min). The cells were then resuspended in 500 µl fixing solution (Paraformaldehyde 4%, Picric acid 0,19% in PBS) and incubated for 30-60 min at RT. The cells were subsequently washed three times with PBS (1200 rpm, 5 min) and permeabilised with PBS/0,1% SDS for 10 min at RT. The three washing steps were repeated and potential unspecific binding of the antibodies was blocked by a 20 min incubation with PBS/10% FCS. The cells were washed once with PBS and incubated with the relevant primary antibody for 60 min at RT. The cells were washed

three times with PBS and incubated with a fluorescent dye-conjugated secondary antibody for 30 min in the dark. The cells were again washed three times with PBS and pipetted onto poly-L-lysine slides, a cover glass was placed on the slide and the samles were analysed using Confocal Microscopy.

Alternatively, for certain applications the cells were transferred to poly-L-lysine slides after fixation and washing. As all cells used were in suspension this was achieved by using cytospins. Briefly, the cells were resuspended in 350 µl PBS and transferred to slides via centrifugation for 5 min at 500 rpm. Cell permeabilisation and staining was then performed as described above.

2.3 Apoptosis assays

2.3.1. Analysis of morphological changes (FSC/SSC analysis)

During apoptotic cell death coordinated morphological changes occur in the nucleus, cytoplasm and at the cell surface. Characteristic changes include loss of cell volume due to loss of water and ions with consequent compaction of the organelles and an increase in cell density. These specific structural changes can be detected via Flow cytometry, thus allowing the differentiation between live, apoptotic and necrotic cells. Apoptotic cells show reduced forward scatter (FCS) corresponding to reduced cell size and increased side scatter (SSC) corresponding to increased granularity. The amount of apoptotic cells within a cell population was determined using a FACScan. The FSC-signal was plotted against the SSC-signal (FSC/SSC) and the number of cells within defined regions , characteristic for certain cell populations, was evaluated.

2.3.2 Propidium Iodide exclusion

The red fluorescence dye Propidium (used as a Propidium Iodide-salt PI) binds unspecifically to dead cells. The propidium cation can enter dead cells which have lost plasma membrane integritry while it is excluded from live cells with intact plasma

membranes (Tanke et.al., 1982). All dead cells (apoptotic and necrotic) are stained and can be analysed by Flow Cytometry.

Briefly, $1x10^5$ cells were resuspended in 100 µl medium and incubated in a 96-well plate in the absence or presence of a cytotoxic agent. In order to determine cell death, the cells were transferred to a 1 ml FACS tube and 100 µl of PI-solution(PBS/5µg/ml PI) were added shortly before measurement in the Flow Cytometer. The samples were kept at 4°C prior to measurement. The light emmitted by the Argon laser excited PI has a wavelength of λ=650 nm and can be measured via the FL2 and FL3 detectors.

2.3.3 Calculation of specific apoptosis

In several experiments the amounts of dead cells were presented as "% specific cell death".

Percentage of specific cell death was calculated according to the formula:

specific cell death (%) =100 * [X - S]/[100 - S].

where X = mean of 3 measurements of experimental cell death

S = mean of 3 measurements of spontaneous cell death

2.3.4 Nicoletti assay

The Nicoletti assay (Nicoletti et.al., 1991) is a flow cytometric method for measuring the percentage of apoptotic nuclei. These can be discriminated from nuclei of living cells due to their hypodiploid DNA content.

Briefly, $5x10^5$ cells were centrifuged and gently resuspended in 250 µl hypotonic fluorochrome solution (PI 50 µg/ml in 0,1% sodium citrate plus 0,1% Triton X-100). The cells were incubated at 4°C in the dark overnight for lysis to occur. The fluorescent dye could then enter nuclei and intercalate into the DNA thus allowing the measurement of the stained nuclear DNA. Apoptotic nuclei could be detected by flow cytometry via

the FL2 detector as a hypodiploid DNA peak in contrast to normal nuclei with a diploid DNA content.

DNA fragmentation into oligonucleosomal subunits and reduction of nuclear DNA content are hallmarks of classical apoptosis. The Nicoletti assay therefore allows the identification of cells undergoing classical apoptosis and discrimination from necrotic cells.

2.3.5 MTT assay

Determination of cell death via the MTT assay is based on the conversion of the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into dark blue formazan crystals. This conversion reflects the activity of the mitochondrial respiratory chain (Mosmann, 1983). Therefore, this assay detects living cells since dead cells cannot carry out the reaction.

10^4 cells were seeded in triplicate in flat-bottom 96-well plates and incubated with the appropriate stimuli. 25 µl MTT solution (PBS/ 5 mg/ml MTT) were added and incubated for 4 h. The reaction was terminated by the addition of 100 µl stop solution (isopropanol/ 5% formic acid) and the formazan crystals were dissolved under vigorous shaking. The absorption of the yellow tetrazolium salt was determined at λ= 550 nm in an Elisa reader. Specific cell death was calculated according to the following folmula:

$$\text{specific cell death (\%)} = 1 - (E/T)$$

$T = A_{550\,nm}$ in the absence of cytotoxic stimulus

$E = A_{550\,nm}$ of unknown sample

2.3.6 Large-scale DNA fragmentation assay

Large-scale DNA fragmentation, i.e chromatin digestion into fragments of approximately 50 Kbp (Susin et.al., 1999) represents an early step of the apoptotic process. These high molecular weight DNA fragments can be visualised by Pulse-field gel electrophoresis (Schwartz and Cantor, 1984).

For the demonstration of large-scale DNA fragmentation $1x10^6$ cells of interest were washed once with PBS. The dried pellets were then incorporated into agarose plugs to avoid shearing of DNA during extraction. The pellets were resuspended in molten, low-melting-temperature agarose and solidified in blocks whose size matched the thickness of the loading slot of the gel. The cells were digested twice with 1 mg/ml proteinase K in NDS buffer (0,5 M EDTA, 10 mg/ml lauroyl sarcosine) for 12h at 50°C. The agarose plugs were then washed in 0,5xTBE and loaded onto a 1% agarose gel followed by elecrophoresis in 0,5xTBE buffer for 24 h at 200 V with a pulse wave of 60 s and 120° angle (Bio-RadCHEF-DR II).

2.3.7 Assessment of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$)

Apoptotic cells exhibit a reduction in mitochondrial transmembrane potential ($\Delta\Psi_m$) of the inner mitochondrial membrane which precedes other apoptosis associated changes like DNA fragmentation chromatin condensation and membrane disruption (Zanzami et.al., 1995). $\Delta\Psi_m$ can be evaluated by using the fluorochrome 3,3´-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Molecular Probes, Inc.). $DiOC_6(3)$ is a cationic dye which binds specifically to mitochondrial membranes with intact transmembrane potential thus giving a high fluorescence signal. Upon disruption of the transmembrane potential however it no longer accumulates in the mitochondria resulting in a reduced fluorescence signal.

$DiOC_6(3)$ was kept as a stock solution (80 mM in DMSO) at RT and used in a final concentration of 40 nM in PBS. $1x10^5$ cells of interest were centrifuged, washed once with PBS and resuspended in 100 µl PBS/ $DiOC_6(3)$. Following incubation for 15 min at 37°C the cells were washed once with chilled PBS and resuspended in 100 µl PBS. The samples were kept on ice until they were measured by flow cytometry (501 nm, FL-1 detector).

2.3.8 Cytochrome C release assay

Apoptosis is accompanied by signs of mitochondrial membrane permeabilization (MMP), including loss of the inner mitochondrial transmembrane potential ($\Delta\Psi m$) and the release of soluble intermembrane proteins via the outer mitochondrial membrane.

MMP results in the tranlocation of cytohrome c (Cyt-c) from the mitochondrial intermembrane space to the cytosol. Thus, localisation of cytochrome c serves as a marker for mitochondrial involvement in apoptosis.

In order to determine the localisation of cytochrome c the mitochondrial fraction was separated from the cytosolic fraction of apoptotic and nonapoptotic cells using the ApoAlert cell fractionation kit (Clontech) according to the manufacturer's protocol. Subsequently cytochrome c localisation was determined in the different fractions by western blot analysis

2.3.9 Induction of cell death via αCD4 and αCXCR4

For the induction of apoptosis via CD4 and CXCR4 10^5 cells were incubated in 60µl of culture medium in the presence or absence of 10µg/ml mAbs against CD4 or CXCR4 or both, for 30 min, at 37°C. The cells were subsequently transferred to a 96 well microtiter plate previously coated with 50µg/ml sheep-anti mouse Ig.

For the investigation of the apoptosis inhibition (chapter 2) PBLs were incubated as before and either CD95- Fc (50 µg/ml), or TNF-R2-Fc (30µg/ml), or TRAIL-R2-Fc (30µg/ml), or the caspase inhibitor BOC-Asp(OMe)-fmk (50µM) were included in the assay. The samples were preincubated for 40 min with the inhibitors and the αCD4/αCXCR4 mAbs before being transferred to sheep anti-mouse Ig coated plates. Apoptosis was assayed after the appropriate incubation time using one of the methods described above.

2.4 Molecular Biology Methods

2.4.1 Preparation of Plasmid DNA

2.4.1.1 Mini Preparation

2 ml of a bacterial cell culture in the exponential growth phase were centrifuged (2 min, 5000 rpm, 20°C) and the pellet was resuspended in 500 µl STET-Buffer (8% Sucrose, 0,5% Triton X-100, 50mM EDTA, 10mM Tris/HCl, pH 8). The bacterial cells were lysed by addition of 50µl lysozyme solution (50mg/ml) for 3 min at RT. They were then icubated for 90 sec at 95°C, in order to induce protein denaturation, and centrifuged (5 min, 13.000 rpm) in order to exclude cell debris. The supernatant was tranferred into a new tube and the plasmid DNA was precipitated after addition of 0,5 ml isopropanol and 50 µl ammonium acetate (8M) and immediate centrifugation (5 min, 13000 rpm). The pellet was then washed with 70% ethanol, air-dried for 10 min and dissolved in 30 µl sterile deionized H_2O.

2.4.1.2 Maxi Preparation

For the preparation of large amounts of plasmid DNA the Qiagen Plasmid Maxi Kit was used according to the manufacturer's protocol.

2.4.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions catalyzed by a DNA polymerase. The DNA to be amplified (template DNA) is first denatured by heating in the presence of the two nucleotides and four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerase. The cycle of denaturation, annealing, and DNA synthesis is then repeated many times. As the products of one round of amplification serve as templates for the next, each successive cycle doubles the amount of the desired DNA product.

Briefly the following standard protocol was used:

5 µl	10x PCR-Buffer
1 µl	dNTPs (10 mM dATP, dGTP, dCTP, dTTP)
1 µl	Primer 1 (100 pmol)
1 µl	Primer 2 (100 pmol)
1-50 ng	Template DNA
1 µl	*Pfu* DNA-Polymerase
in 50 µl	reaction volume (adjusted by addition of H_2O)

The reaction was overlayed with 50 µl mineral oil and run with the following temperature profile:

Start	Initial Denaturation	4 min at 94°C
25-35 Cycles	Denaturation	1 min at 94°C
	Annealing	1 min at 56°C
	Elongation	1 min at 72°C
Termination		5 min at 72°C

The annealing temperature for specific sets of primers was calculated as follows:

$$Temp = 2x(A \text{ or } T) + 4x(G \text{ or } C) - 4°C$$

2.4.3 DNA restriction cleavage

The DNA restriction cleavage reactions were carried out by using 1-2 µg of plasmid DNA, prepared via a mini or maxi plasmid preparation. 2 U of an appropriate restriction endonuclease were added and the reaction was performed in the presence of an enzyme specific reaction buffer provided by the manufacturer. The reaction was

incubated for at least 1 h at 37°C. The resulting restriction fragments were analysed by agarose gel electrophoresis.

2.4.4 Agarose Gel Electrophoresis

Following digestion of DNA with restriction enzymes, the DNA fragments can be separated and identified via electrophoresis through an agarose gel.

Briefly, 1xTBE buffer was used for the preparation of the gel and as running buffer. Depending on the size of the DNA fragments to be resolved 0,7-2 % agarose gels were used. Fragments between 0.8 and 12 Kb were analysed in a 0.8 % agarose gel while fragments between 0.5 and 2 Kb in a 2% agarose gel. After the addition of DNA sample buffer the samples were loaded on the gel and run for 60-120 min at 80 V. A DNA molecular weight marker, containing a mixture of fragments of known sizes, was also loaded on the gel.

Following electrophoresis the gel was incubated for 15 min in TBE/0.5 μg/ml ethidium bromide and washed 3 times for 5 min with H_20.

The fluorescent dye ethidium bromide intercalates unspecifically into double-stranded DNA molecules thus allowing the detection of the DNA fragments by direct examination of the gel in ultraviolet light.

2.4.5 DNA extraction from Agarose Gels

The extraction of DNA fragments for agarose gels was performed by using the Qiagen Gel-Extraction kit according to the manufacturer's protocol.

2.4.6 Vector dephosphorylation

Restriction enzyme digested plasmid DNA should be dephosphorylated prior to use in a ligation reaction. Dephosphorylation prevents recircularization of the linearized vector without insertion of the foreign DNA.

Briefly, 10 U of Calf Intestinal Alkaline Phosphatase (CIAP) were added to a restriction reaction followed by a further incubation for 45-60 min at 37°C.

2.4.7 DNA Ligation

Ligation of fragments of foreign DNA of interest to plasmid vectors was performed by using a 1:3 concentration ratio of vector to insert. The ligation reaction had the following composition:

15 µl Vector +Insert (adjusted by addition of H_2O)
2µl 10 mM Li [ATP]
2µl 10x Ligation buffer
1µl T4 DNA Ligase

The ligation reaction was incubated either for 3- 4 h at RT or overnight at 16°C.

2.4.8 Tranformation

Amplification of plasmid DNA is achieved via transformation in bacteria. Competent *E.coli* XL1 and TOP 10 F bacterial strains were used for transformation.

Briefly, 50 µl of competent bacteria were thawed on ice. 1-5 µl DNA (100-200 ng) were added, followed by a 30 min incubation on ice. The cells were then heated at 42 °C for 95 sec and then chilled on ice for 2 min, thus inducing permeabilization of the bacterial cell wall and uptake of foreign DNA molecules. 900 µl of antibiotic free LB medium were added and the cells were incubated for 30-45 min at 37°C in order to induce antibiotic resistance. The cells were then plated onto antibiotic containing LB-agar and incubated overnight at 37°C to allow selection of bacteria carrying the plasmid of interest. Several bacterial colonies were subsequently picked and the plasmid DNA was amplified and purified via a Mini Preparation (see II.2.4.1). A restriction analysis was then performed and positive clones, containing the plasmid of interest, were used to produce larger amounts of DNA via a Maxi Preparation (see II.2.4.2).

2.4.9 Stable transfection of eukaryotic cells via electroporation

Expression of proteins of interest in eukaryotic cells was achieved via transfection of the respective plasmid DNA. The DNA was transfected to the cells via electroporation (Neumann et. al., 1982). Briefly, $5x10^6$ cells were centrifuged (10 min, 1200 rpm), resuspended in 400 µl culture medium and transferred to an electroporation cuvette. 10-30 µg of plasmid DNA were added, gently mixed and the cells were electroporated at 960 µF and 220 V. The cells were immediately transferred to a 6-well plate and resuspended in 6 ml warm culture medium. Following a 48 h incubation the cells were counted, centrifuged and resuspended in selection culture medium (see 2.1.1). The cells were then transferred to 96 well plates at a density ranging from 10^3 to 10^4 cells per well. 2-3 weeks later single cell colonies were seen in the microtiter plates. These were tested for the expression of the protein of interest via immunofluorescence staining (see 2.2.3.1).

2.5 Biochemical Methods

2.5.1 Preparation of eukaryotic cell lysates

$1x10^6$ cells per sample were incubated with the appropriate stimuli, centrifuged in a 50 ml tube (5 min, 1200 rpm , RT) and washed once with 50 ml PBS. The cells were resuspended in 1 ml PBS transferred to an eppendorf tube and centrifuged (5 min, 4000 rpm, 4°C). The supernatant was discurded, the cell pellet was resuspended in lysis buffer (1ml lysis buffer/ 10^7 cells) and incubated for 20 min at 4°C. Following centrifugation (15 min, 13000 rpm, 4°C) the supenatant was transferred to a new tube and the protein concentration was determined. 6 µl reducing sample buffer were added and the samples were incubated for 3 min at 95°C. The samples were then analysed by polyacrylamide gel electrophoresis.

2.5.2 Protein Concentration Assay

The determination of protein concentration was performed by using the BCA protein assay.

Bicinchoninic acid (BCA) builds a highly specific violet complex with Cu^{+1} which is formed when Cu^{+2} is reduced by protein in an alkaline environment. This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. The advantage of this method is that it is compatible with detergents thus allowing the determination of protein concentration of cell lysates.

Briefly, 1 ml BCA reagent (1:50 $CuSO_4$:BCA) was added to 50 µl protein solution (5 µl lysate + 45 µl PBS). The reaction was incubated for 30 min at 37°C and then measured in a spectrophotometer at λ=562 nm.

The protein concentration of the sample is determined by comparison to a standard concentration versus absorbance curve prepared by using a dilution series of BSA in PBS.

2.5.3 Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

The denaturing polyacrylamide gel electrophoresis was used for the separation of proteins according to their molecular weight. The method used was the discontinuous gel electrophoresis originally described by Laemmli, 1970.

7,5-15 % acrylamide running gels and 5 % acrylamide stacking gels were used. The gels had the following dimensions:

Stacking gel: 233 mm x 12 - 27 mm x 1.5 mm

Running gel: 233 mm x 203 mm x 1.5 mm

The gel composition is shown in II.1.2. Polymerization of the gels was initiated by the addition of 0.1 % (v/v) TEMED and the polymerising solution was immediately used. The running gel was overlayed with isopropanol and left to polymerize for 30 min. The isopropanol was then washed away with water and the remaining liquid was removed with filter paper. The gel was then cast with the stacking gel which was left to polymerize for 10 min. The gel was then loaded and run at 80-200 V for 8-18 h.

2.5.4 ^{35}S labelling of eukaryotic cells

For the radioactive labelling of newlly synthesized proteins cells, in the exponential growth phase, were incubated in defficient RPMI medium (see II.1.4.1) for 1 h at 37°C. The medium was subsequently supplemented with ^{35}S labelled cysteine and methionine. 1 mCi ^{35}S-cysteine/methionine (Pro Mix, Amersham) per 3 x 10^7 cells was used. The cells were incubated for 24 h at 37°C and were then washed once in PBS. The samples were either stimulated with appropriate antibodies or left untreated and were analysed by one dimensional gel electrophoresis (see II.2.5.3). Following electrophoresis the gel was dried for 2 h at 80°C and was placed under an X-ray sensitive film (X- OMAT AR, Kodak).

2.5.5 Immunoprecipitation

1x10^7 unstimulated or stimulated cells were lysed as described in II 2.5.1 . Samples were clarified by centrifugation at 14000 rpm for 15 min at 4°C. Insoluble nuclei formed a pellet which was avoided as the supernatant was removed. 20 µl of mouse IgG coupled protein A-sepharose beads were added to the clarified supernatants and incubated for 1 h at 4°C on a tumbler in order to reduce unspecific binding of proteins to the immunoprecipitating antibody (control immunoprecipitation).

The samples were centrifuged and the precleared lysates were incubated with 20 µl protein-G-sepharose beads and appropriate antibodies against the antigens of interest. The samples were incubated for 1-4 h at 4°C on a tumbler and then washed 5 times with 1 ml lysis buffer (1 min, 6000 rpm). The beads were then dried using a Hamilton syringe and 40 µl reducing sample buffer were added prior to boiling for 5 min at 95°C. The samples were then analysed by gel electrophoresis.

2.5.6 Western Blot

Proteins were transferred from polyacrylamide gels to nitrocellulose membranes by electroblotting (Towbin et. al., 1979).

Semi-dry transfer:

All elements used were presoaked in transfer buffer (see II.1.2). The gel and membrane (Hybond-ECL membrane C, Amersham-Buchler, Braunschweig) were placed between sheets of absorbant paper (4 on each side). The graphite electrodes of the blotting chamber were also humidified with transfer buffer and the blot was run at 0.8 mA/cm^2 for 90 min at RT.

Wet transfer:

The gel and membrane were placed between sheets of absorbant paper (3 on each side) and were immersed in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) in a tank blot. The blot was run at 500 mA for 90 min at 4°C.

Following transfer, non specific binding sites on the nitrocellulose membrane were blocked by incubation with 5% (w/v) milk powder or 5% (w/v) BSA in TPBS, for 1 h at RT. Membranes were washed 3 times for 10 min with TPBS. Appropriate primary antibodies were diluted in TPBS and incubated for 1 h at RT or overnight at 4°C, with constant shaking. Membranes were washed 3 times for 10 min with TPBS. Detection horseradish peroxidase (HRP) conjugated antibodies were diluted 1:20 000 in TPBS and incubated with the membranes for 45 min with gentle shaking. Membranes were washed a further 3 times with TPBS.

Detection of proteins was achieved by a 2 minute incubation with the ECL reagent (Rennaissance-Kit, NEN, Bad Homburg v.d.H). HRP catalyses the oxidation of the ECL substrate luminol in the presence of hydrogen peroxide, which leads to the emission of light. Excess ECL reagent was removed and the membrane was placed under an X-ray sensitive film (X- OMAT AR, Kodak). Films were processed through an X-ray developer at various exposure time points.

Blot stripping

Antibody complexes can be removed from nitrocellulose membranes which can then be reprobed for different proteins. Membranes were incubated in "Stripping buffer" (62.5 mM TrisHCl pH=8. 2% SDS, 100 mM ß-Mercaptoethanol) for 30 min, at 56°C.

Membranes were washed 6 times with TPBS for 10 min at RT. The membranes were then blocked and incubated with appropriate antibodies as described above.

2.6 Statistical methods

The Wilcoxon Rank-sum test (Lehmann, 1975) was used for the comparison of distributions of two independent samples. The shift in location was estimated by the Hodges-Lehmann estimate together with the 95% confidence interval for the shift parameter. If more than two groups had to be compared the Kruskall-Wallis test (Lehmann,1975) was applied.

A cutpoint analysis of specific apoptosis was performed using a maximally selected test statistic according to the test procedure by Miller & Siegmund (1982). Possible cut point estimates were tested in the midth 80% of the specific apoptosis values obtained with the different stimuli. The χ^2 test was used to calculate the statistic and P value for each specific apoptosis value considered. The corrected P value was then calculated according to Miller & Siegmund for the maximum of the χ^2 statistic. Cut point estimates with corrected P values<0.05 were considered statistically significant. 95% bootstrap confidence sets were computed according to Dümbgen (1991). To analyze the effect of therapy on the specific apoptosis a linear regression analysis was performed.

III RESULTS

1 αCD4, αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals.

Previous studies have shown that the HIV-1 surface glycoprotein gp120 induces apoptosis in T cells via CD4 and recently also via CXCR4 (Berndt et al., 1998). In these experiments it was shown that both receptors can mediate a "novel" type of caspase independent apoptosis. The experiments presented here were designed in order to investigate whether this novel αCD4 and αCXCR4 induced apoptosis is relevant to the pathophysiology of HIV-1 infection. Susceptibility of PBLs from HIV-1 infected individuals to CD4/CXCR4 mediated apoptosis was assessed in comparison to that of PBLs from healthy individuals. Subsequently the features of αCD4/αCXCR4 induced apoptosis in HIV-1 infected PBLs were investigated.

1.1 Patient characterisation

The HIV-1 positive individuals who took part in this study were recruited from the Department of Internal Medicine I, Mannheim Medical School, University of Heidelberg and from the private practice of Drs. Schuster/Brust in Mannheim. The study was approved by the Ethics Committee II of the University of Heidelberg and performed in accord with the Declaration of Helsinki. An informed consent was obtained from all participants. All HIV-1 infected individuals were classified according to the CDC, 1993, and were older than 18. The clinical and immunological characteristics of the participants are shown in Table III.1. Exclusion criteria were endocrinological diseases, liver cirrhosis, serum creatinine higher than 1.5 mg/dl, cardiorespiratory insufficiency, alcohol or drug abuse. As a control group, healthy blood donors were recruited from the staff of the collaborating institutions.

	HIV-1$^-$	HIV-1$^+$
No. of patients	78	202
Age (years)[1]	31.2 ± 9.24	39.3 ± 9.9
Sex (F/M)	32/46	65/137
Viral load (log copies/ml) [1]	ND	4.6 ±5.15 [(4)]
CD4$^+$ cells (cells/µl) [1]	ND	439.3 ± 247.5
CD8$^+$ cells (cells/µl) [1]	ND	1017.1 ± 595.9
CD4$^+$/ CD8$^+$ ratio [1]	ND	0.42 ± 0.32
Treatment [2]	_	Naive (n=52) ART, double (n=31) ART, triple (n=49) HAART, triple+PI (n=70)
Stage (CDC 1993) [3]	_	A1/A2 (n=7/3) B1/B2/B3 (n=41/114/18) C1/C2/C3 (n=1/1/17)

Table III.1: Patient characteristics. [1] numbers are mean ± SD [2]ART: antiretroviral therapy; double, triple: combinations of two or three nucleoside or non-nucleoside analogues, HAART: highly active ART, PI: protease inhibitor [3] CDC centres of disease control, classification of 1993 in [4] n=28 patients the viral load was below 50 copies/mL (detection limit), bDNA Test (Chiron company)

1.2 Increased susceptibility of PBLs from HIV-1 infected individuals to αCD4 and αCXCR4 induced apoptosis

The sensitivity of PBLs from HIV-1 infected individuals to αCD4 and αCXCR4 induced apoptosis in comparison to PBLs from healthy controls was examined. In order to mimick gp120 binding and to activate both receptors individually mAbs against CD4 (HP2/6) or CXCR4 (12G5) were used. The mAbs used interfere with binding of gp120 as determined by surface staining or inhibit HIV-1 infection, respectively. Moreover, in order to evaluate whether stimulation via both CD4 and CXCR4 shows an additive effect, PBLs were also stimulated with both antibodies simultaneously.

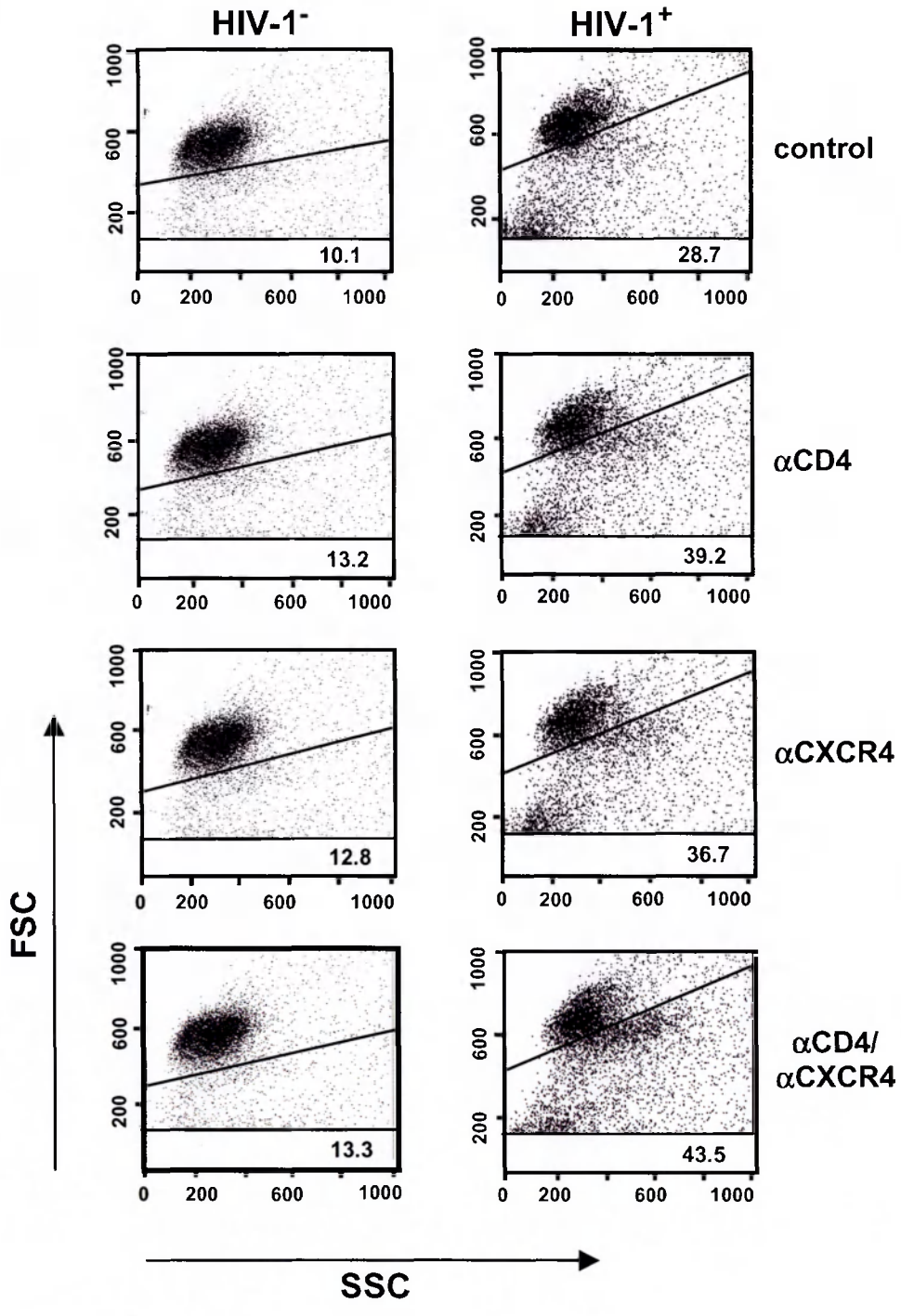

Fig. III.1: CD4 and CXCR4 mediate apoptosis in PBLs from HIV-1 infected individuals. FSC/SSC analysis of apoptosis in PBLs from a representative HIV-1 negative (left panels) and a HIV-1 positive individual (right panels). 10^5 cells were incubated in the presence or absence of 10µg/ml αCD4 or αCXCR4 or both for 30 min at 37°C and were then transferred to a 96 well plate previously coated with 50 µg/ml sheep anti-mouse immunoglobulin. Percentages of apoptotic cells are indicated in the lower right corner.

PBLs from an HIV-1 infected or a healthy individual were incubated in the presence or absence of either αCD4 or αCXCR4 or both. Apoptosis was induced as described in II.2.3.9 and was assessed 24 h following stimulation. Apoptosis was determined via FSC/SSC analysis, as morphological criteria provide a reliable method of evaluation of the previously described "novel" type of cell death as well as classical apoptosis. Moreover this method requires little patient material.

Freshly isolated PBLs from an HIV-1$^-$ individual showed low levels of spontaneous apoptosis after 24 h incubation in RPMI medium and were resistant to apoptosis induced via CD4 and CXCR4. PBLs from an HIV-1$^+$ individual showed high levels of spontaneous apoptosis as it has been previously described (Gougeon et al., 1993) as well as susceptibility to CD4 and CXCR4 mediated cell death (figure III.1). Each receptor could individually mediate apoptosis while engagement of both receptors showed an additive effect. These data suggested a role for CD4/CXCR4 mediated apoptosis in HIV-1 induced T cell depletion.

1.3 Kinetics of αCD4 and αCXCR4 induced apoptosis

Initially αCD4/αCXCR4 induced apoptosis was determined after 24 h. However, the "novel" apoptosis previously described in the T cell line HPBALL (Lipoldova et al., 1989) and in the CD4 and CXCR4 transfected B cell line BJAB showed rapid kinetics. The apoptotic process was complete as early as 2h following stimulation. In order to determine whether similar kinetics applied to primary cells from HIV-1 infected individuals, apoptosis was evaluated at several time points after stimulation.

Freshly isolated PBLs from an HIV-1 infected individual or a healthy control were incubated in the presence or absence of αCD4 or αCXCR4 as described in II.2.3.9. Apoptosis was evaluated by FSC/SSC analysis at the time points indicated in figure III.2.

In contrast to cell lines, no apoptosis induction via CD4 or via CXCR4 was observed at early time points. Apoptosis induction was initiated approximately 8 h following stimulation with a maximal effect observed after 18 h. Further incubation for 24 or 48 h did not alter the levels of apoptotic cells. Therefore apoptosis was determined after 18 h in all further experiments.

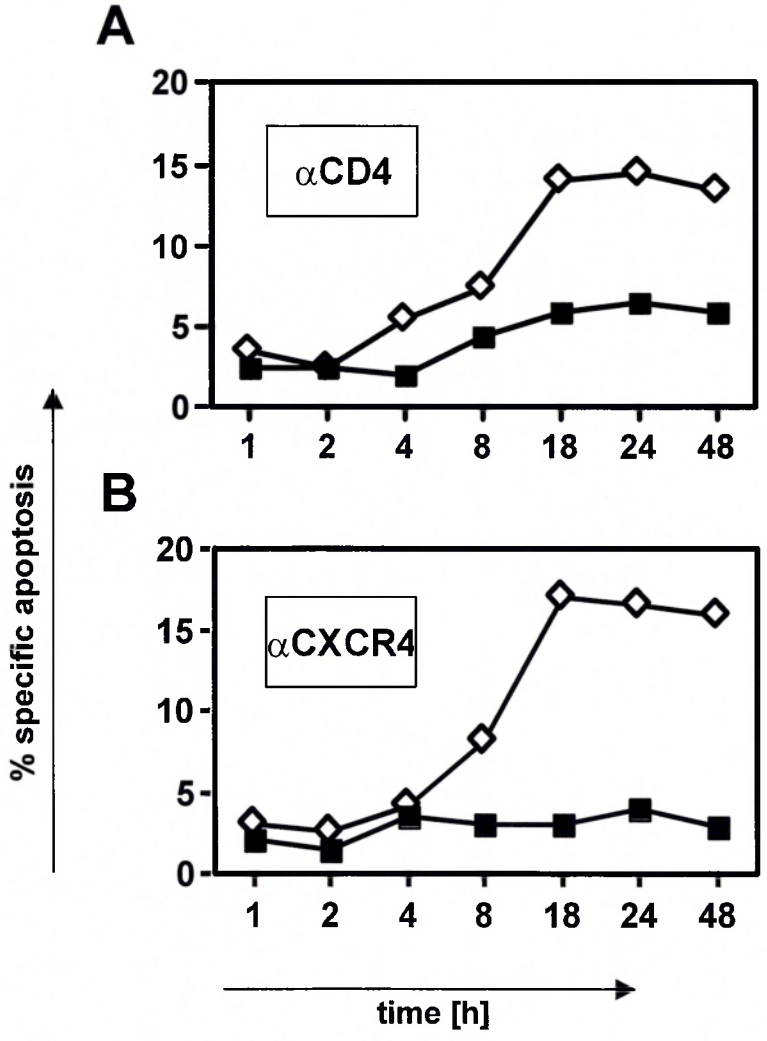

Fig. III.2: Kinetics of αCD4 and αCXCR4 induced cell death. Kinetic analysis of apoptosis induced by αCD4 (A), or αCXCR4 (B) in PBLs from a HIV-1^+ (■) and a HIV-1^- (◊) individual. Cell death was determined by FSC/SSC analysis at the indicated time points. Specific apoptosis was calculated as described in Materials and Methods (see II.2.3.3). One representative experiment of 10 performed in triplicates is shown.

1.4 Evaluation of CD4 and CXCR4 mediated apoptosis in a representative cohort of HIV-1 infected individuals

Since the initial findings suggested that PBLs from HIV-1 infected individuals were sensitive to αCD4/αCXR4 induced apoptosis, a larger number of patients was examined. However, induction of CD4 and CXCR4 mediated apoptosis showed high variability between different HIV-1 infected individuals. Following an initial evaluation of apoptosis induction in 50 HIV-1 infected individuals and 30 healthy controls, «cutpoint» estimates of specific apoptosis values were calculated (Miller & Siegmund, 1982). These values represented limits above which apoptosis induction was considered statistically significant (see II.2.6). The following values were determined for CD4, CXCR4 and CD4/CXCR4 induced apoptosis:

Stimulus	Cutpoint estimate (% specific apoptosis)	95% Confidence Interval
αCD4	4.6	1.8 to 7.3
αCXCR4	3.9	1.1 to 7
αCD4+αCXCR4	5.3	3.2 to 7.7

Therefore in the overall patient cohort, which comprised 202 individuals, only apoptosis values higher than the cutpoint estimates were considered.

In the overall group of individuals examined, specific αCD4, αCXCR4 and αCD4/αCXCR4 triggered cell death was significantly higher in PBLs from HIV-1$^+$ than from HIV-1$^-$ individuals with P values < 0.001 in all cases (figure III.3). αCD4 treatment induced 5.3 % specific apoptosis (mean, CI_{95} 3.9 to 6.6) and αCXCR4 treatment induced 5. 53 % specific apoptosis (mean, CI_{95} 3.8 to 7.2) in HIV-1$^+$, n= 202, versus 1.76 % (mean, CI_{95} 0.94 to 2) and 0.11% (mean, CI_{95} -1 to 0.73), respectively in HIV-1$^-$ individuals, n= 78. Triggering of both CD4 and CXCR4 receptors induced 6.96 % apoptosis (mean, CI_{95} 5.7 to 8.25) in HIV-1$^+$ versus 0,89 % (mean, CI_{95} 0.05 to 1.7) in HIV-1$^-$ subjects.

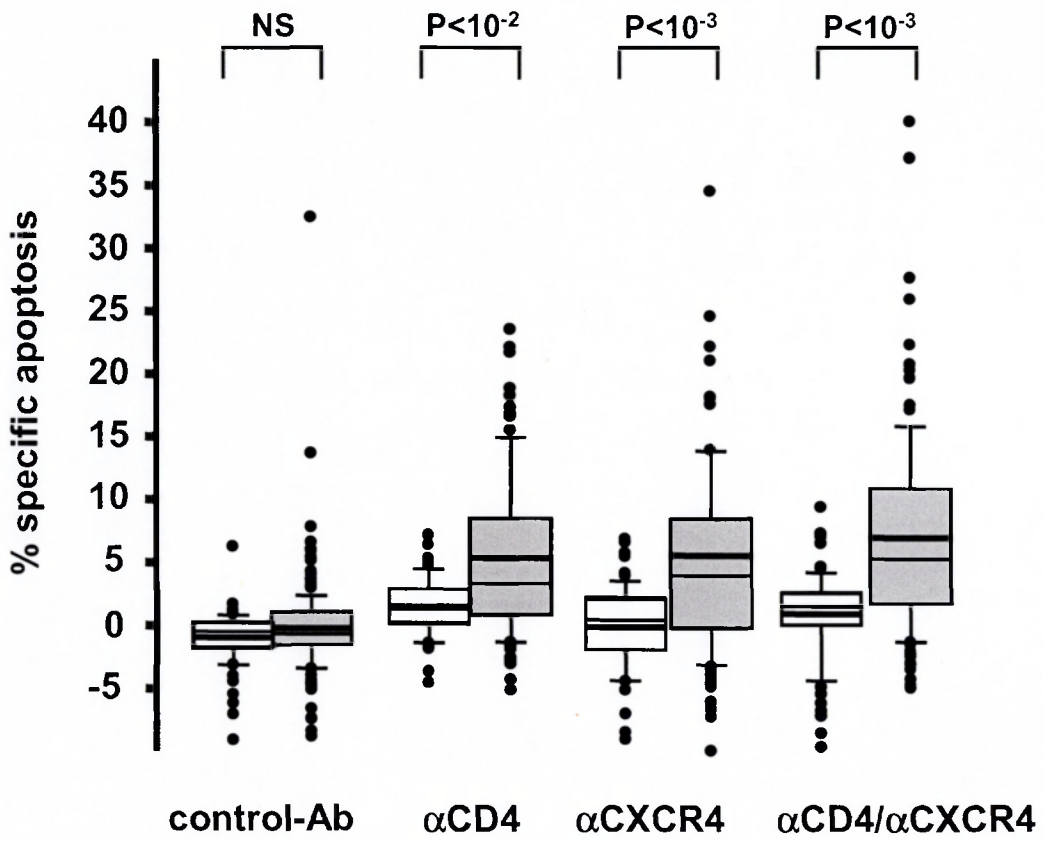

Fig. III.3: CD4/CXCR4 mediated apoptosis in a cohort of HIV-1 infected individuals. αCD4, αCXCR4 and αCD4/αCXCR4 induced apoptosis is significantly higher in PBLs from 202 HIV-1$^+$ individuals (shaded plots) than from 78 healthy control subjects (blank plots) (P values < 0.001). Apoptosis was determined by FSC/SSC analysis 18 h after incubation with mAbs against the receptors. Specific apoptosis was calculated as described in Materials and Methods (II.2.3.3). Thin lines represent the median while thick lines represent the mean.

1.5 Effect of clinical and immunological parameters on apoptosis induction

Since a high variability was observed in apoptosis induction between different HIV-1 infected individuals, possible correlations with clinical and immunological characteristics of the patients were assessed. It was hypothesized that patients with high

viral load and low CD4 counts show higher levels of CD4/CXCR4 mediated apoptosis. The Wilcoxon rank sum test was used to evaluate the effect of CD4 counts, viral load, treatment and stage of the disease of the HIV-1 infected individuals on the induction of apoptosis. The Kruskall Wallis test was used to evaluate the effect of the therapy regimen. In the overall group of patients no statistically significant correlation could be established between any of these parameters and levels of PBL apoptosis. Interestingly, however, a statistically significant correlation ($P<0.05$) was found between high viral load (above 5000 copies/ml) and high levels of CXCR4 and CD4/CXCR4 induced apoptosis in patients which were not under treatment (therapy naive).

1.6 HAART leads to a decrease in αCD4/αCXCR4 induced apoptosis

It has been previously reported that HAART therapy decreases apoptosis in lymphoid tissue and in PBLs of HIV-1 infected individuals (Badley et al., 1998; Badley et al., 1999). Although no correlation could be established between apoptosis induction and therapy regimen per se, the effect of therapy initiation/change on CD4/CXCR4 apoptosis was investigated. Within the timeframe of the study, the induction of αCD4/αCXCR4 apoptosis was repeatedly assessed in PBLs from some individuals (n=28). The characteristics of this patient subgroup are shown in table III.2. These individuals could be subdivided into two groups, group A: subjects with no change in their therapy regimen between the different measurements (n=19), group B: subjects that were either initiated into therapy (ART or HAART) or had a change/addition in one or more components of their therapy regimen (n=9). In order to evaluate the effect of change in therapy on specific apoptosis a linear regression analysis was performed. As shown in figure III.4 initiation to HAART regimen significantly reduced, but did not abrogate, αCXCR4 and αCD4/αCXCR4 induced apoptosis ($P<0.05$).

	HIV-1$^+$ (repeated patients)
No. of patients	28
Age (years)[1]	38.3 ± 9.6
Sex (F/M)	12/16
Viral load (log copies/ml)[1]	4.66 ± 5 [(4)]
CD4$^+$ cells (cells/µl)[1]	438.5 ± 217.3
CD8$^+$ cells (cells/µl)[1]	1088.6 ±593.4
CD4$^+$ / CD8$^+$ ratio[1]	0.46 ± 0.28
Treatment[2]	Naive (n=10)
	ART, double (n=3)
	ART, triple (n=2)
	HAART, triple + PI (n=13)
Stage (CDC 1993)[3]	A2 (n=2)
	B1/B2/B3 (n=4/17/3)
	C1/C2 (n=1/1)

Table III.2 : Characteristics of repeated patients. [1] numbers are mean ± SD [2]ART: antiretroviral therapy; double, triple: combinations of two or three nucleoside or non-nucleoside analogues, HAART: highly active ART, PI: protease inhibitor [3] CDC centres of disease control, classification of 1993 in [4] n=2 patients the viral load was below 50 copies/mL (detection limit), bDNA Test (Chiron company)

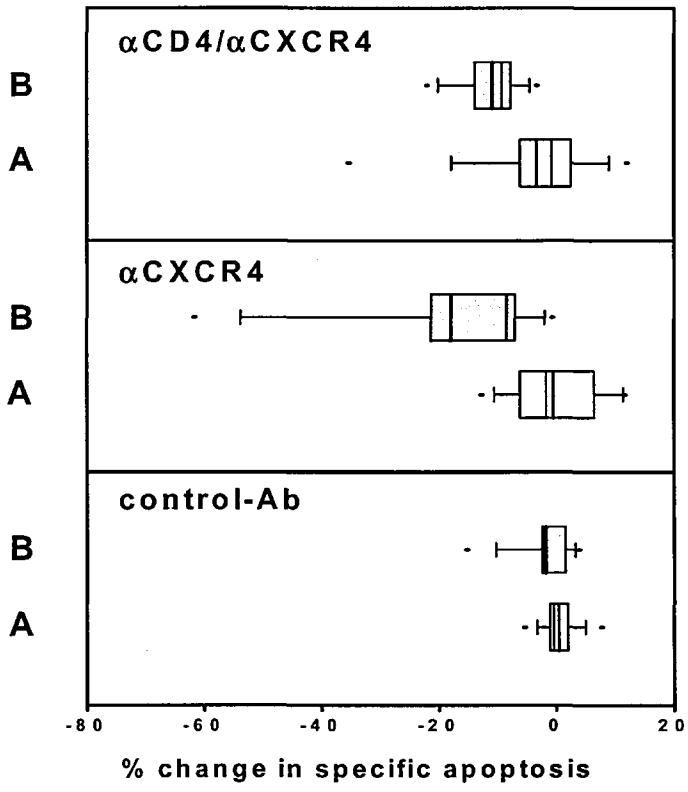

Fig. III. 4 : Effect of therapy change on αCD4/αCXCR4 induced apoptosis. Percentages of change in the induction of specific apoptosis after control Ab (crosslinking Ab), CXCR4 or CD4/CXCR4 stimulation in two groups of HIV-1^+ individuals. Group A (blank plots) represents patients with no change in therapy , Group B (shaded plots), patients initiated into a more complex therapy. Thin lines represent the median while thick lines represent the mean.

2 Features of αCD4/αCXCR4 induced apoptosis

2.1 Role of the CD95-, TNF- and TRAIL- systems in αCD4/αCXCR4 mediated apoptosis

αCD4 and αCXCR4 induced apoptosis in HPBALL and BJAB cells was found to be independent of the CD95, TNF and TRAIL death systems. The HPBALL cell line, however, is insensitive to CD95 and TNF mediated apoptosis. Moreover the rapid kinetics of apoptosis induction could be limiting for the upregulation of the ligands of these receptors.

	HIV-1$^+$ (Study of apoptosis inhibition)
No. of patients	40
Age (years)[1]	43.7 ± 10
Sex (F/M)	8/32
Viral load (log copies/ml) [1]	4.42 ± 4.94 [(4)]
CD4$^+$ cells (cells/μL) [1]	544 ± 269.4
CD8$^+$ cells (cells/μL) [1]	1083 ± 449.3
CD4$^+$/ CD8$^+$ ratio [1]	0.57 ± 0.4
Treatment [2]	Naive (n=7) ART, double (n=4) ART, triple (n=24) HAART, triple + PI (n=5)
Stage (CDC 1993)[3]	A1 (n=2) B1/B2/B3 (n=13/21/3) C3 (n=1)

Table III.3: Patient characteristics. numbers are mean ± SD [2]ART: antiretroviral therapy; double, triple: combinations of two or three nucleoside or non-nucleoside analogues, HAART: highly active ART, PI: protease inhibitor [3] CDC centres of disease control, classification of 1993 in [4] n=3 patients the viral load was below 50 copies/mL (detection limit), bDNA Test (Chiron company)

The CD95, TNF and TRAIL systems have been suggested to play a role in T cell apoptosis during HIV-1 infection. It was therefore investigated whether the αCD4, αCXCR4 induced apoptosis observed in PBLs from HIV-1 infected individuals was dependent on these receptor systems. PBLs from 40 patients were used for these experiments (for patient characteristics see table III.3).

Initially both αCD4 and αCXCR4 induced apoptosis were examined and similar results were obtained for both receptors. However, due to limitations in patient material apoptosis was induced by αCD4+αCXCR4 in these experiments.

2.1.1 αCD4/αCXCR4 induced apoptosis is independent of the CD95 system

In order to investigate the role of CD95 in αCD4/αCXCR4 mediated apoptosis an inhibitor of the CD95 system , CD95-Fc, was used (Dhein et al., 1995) (see II.1.8). PBLs were incubated in the presence or absence of CD95-Fc for 40 min at 37°C, followed by stimulation with αCD4 and αCXCR4 (see II.2.3.9). As a control, Jurkat cells were preincubated with CD95-Fc and apoptosis was induced via incubation with 1 µg/ml αApo1. Jurkat cell apoptosis was measured after 10 h while apoptosis of PBLs after 18 h, via FSC/SSC analysis.

In the overall group of HIV-1 infected individuals tested specific αCD4/αCXCR4 induced apoptosis was not significantly inhibited by CD95-Fc (P=0.17) (figure III.5). In contrast CD95 mediated apoptosis was entirely blocked by CD95-Fc in Jurkat cells thus demonstrating the functionality of the inhibitor.

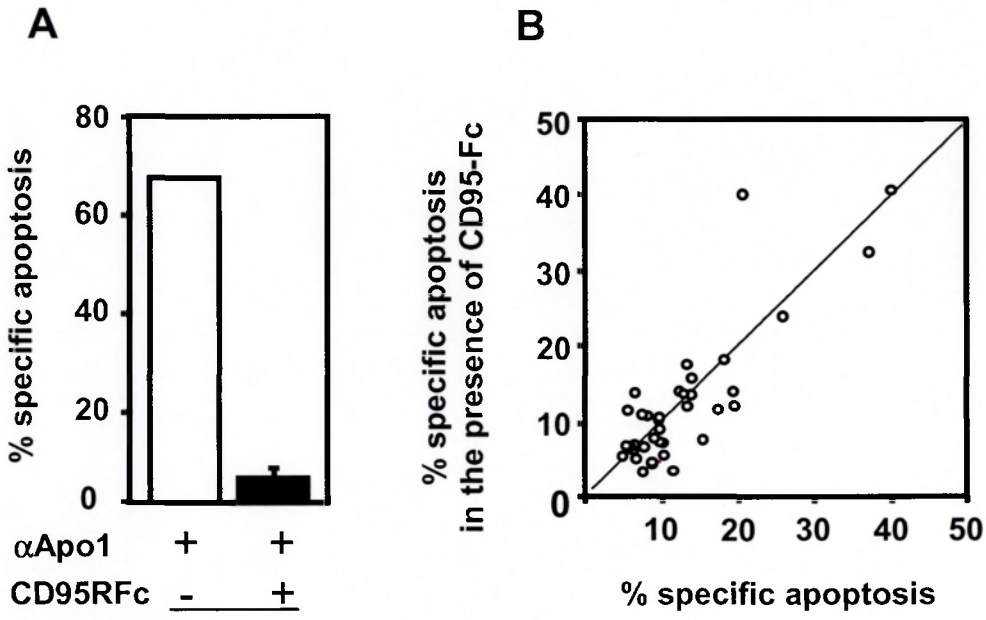

Fig. III.5: αCD4/αCXCR4 induced apoptosis is independent of the CD95 system.
(A) Specific αApo1 induced apoptosis in Jurkat cells in the presence (black bars) or absence (white bars) of 10 μg/ml CD95-Fc. The experiment was performed in triplicates and the results are presented as mean±SD.
(B) Specific αCD4/αCXCR4 induced apoptosis, in PBLs from 40 HIV-1 infected individuals, in the presence (Y axis) or absence (X axis) of 50 μg/ml CD95-Fc.

2.1.2 αCD4/αCXCR4 induced apoptosis is independent of the TNF system

The TNF system has been suggested to contribute to apoptosis induced by CD4 (Han et al., 1996) and recently also via CXCR4 (Herbein et al., 1998). In order to examine the contribution of the TNF system in the present experimental system the TNF-R2-Fc construct was used which inhibits receptor-ligand interactions.

Apoptosis was induced in PBLs from 40 HIV-1 infected individuals as previously described with an additional preincubation in the presence or absence of TNF-R2-Fc (see II. 2.3.9).

As a control for the activity of the inhibitor the murine cell line L929 was used which can be sensitized to TNFα induced cell death in the presence of Actinomycin D. $2x10^4$

L929-cells were incubated with 1 μg/ml actinomycin D for 24 h at 37°C, followed by a further 30 min incubation in the presence or absence of TNF-R2-Fc and further addition of 10 ng/ml TNFα.

Apoptosis of PBLs from HIV-1 infected patients was measured after 18 h by FSC/SSC analysis while L929-cell apoptosis was determined after 24 h using the MTT test. In the overall group of patients tested no significant inhibition of αCD4/αCXCR4 induced apoptosis was observed (figure III.6). In contrast, TNFα induced apoptosis of L929 cells was entirely blocked thus demonstrating the functionality of the construct used.

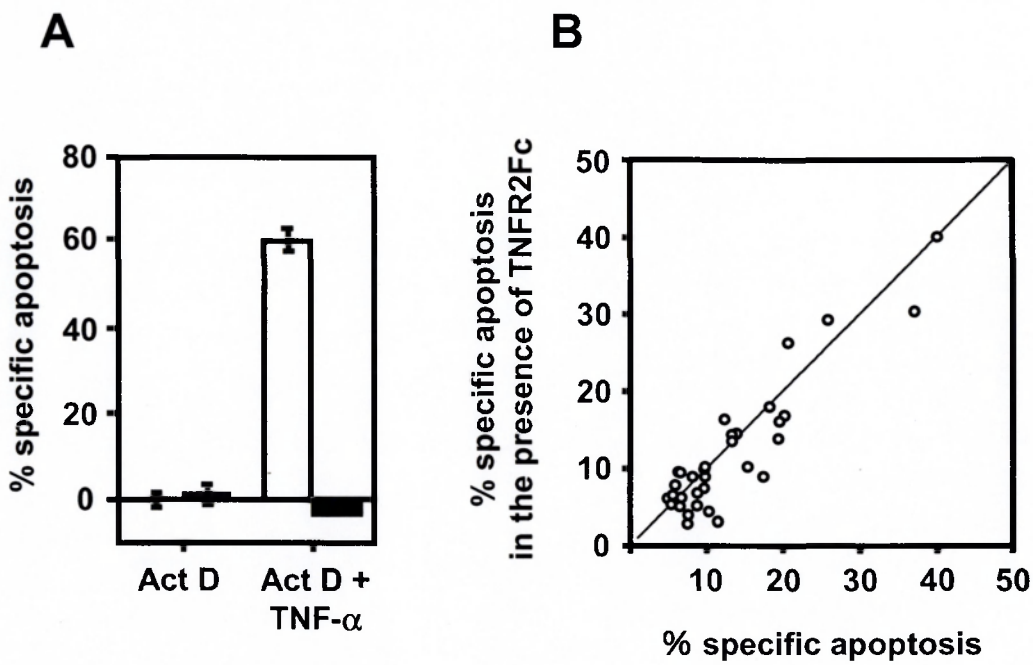

Fig. III.6: αCD4/αCXCR4 induced apoptosis is independent of the TNF system. (A) Specific TNFα induced apoptosis in L929 cells in the absence (white bars) or presence (black bars) of 10 μg/ml TNF-R2-Fc. The experiment was performed in triplicates and the results are presented as mean±SD. (B) Specific αCD4/αCXCR4 induced apoptosis, in PBLs from 40 HIV-1 infected individuals, in the presence (Y axis) or absence (X axis) of 30 μg/ml TNF-R2-Fc.

2.1.3 αCD4/αCXCR4 induced apoptosis is partially dependent on the TRAIL system

Recent data have also suggested a potential role for TRAIL in apoptosis in HIV-1 infected patients (Katsikis et al., 1997; Jeremias et al., 1998). The contribution of the TRAIL system in αCD4/αCXCR4 induced apoptosis was evaluated by using the TRAIL-R2-Fc inhibitor. Apoptosis of PBLs from HIV-1 infected individuals was induced as previously described (II.2.3.9) in the presence or absence of TRAIL-R2-Fc. As a control, Jurkat cells were incubated overnight with 0.5 μg/ml leucine zipper TRAIL (LZT), in the presence or absence of TRAIL-R2-Fc.

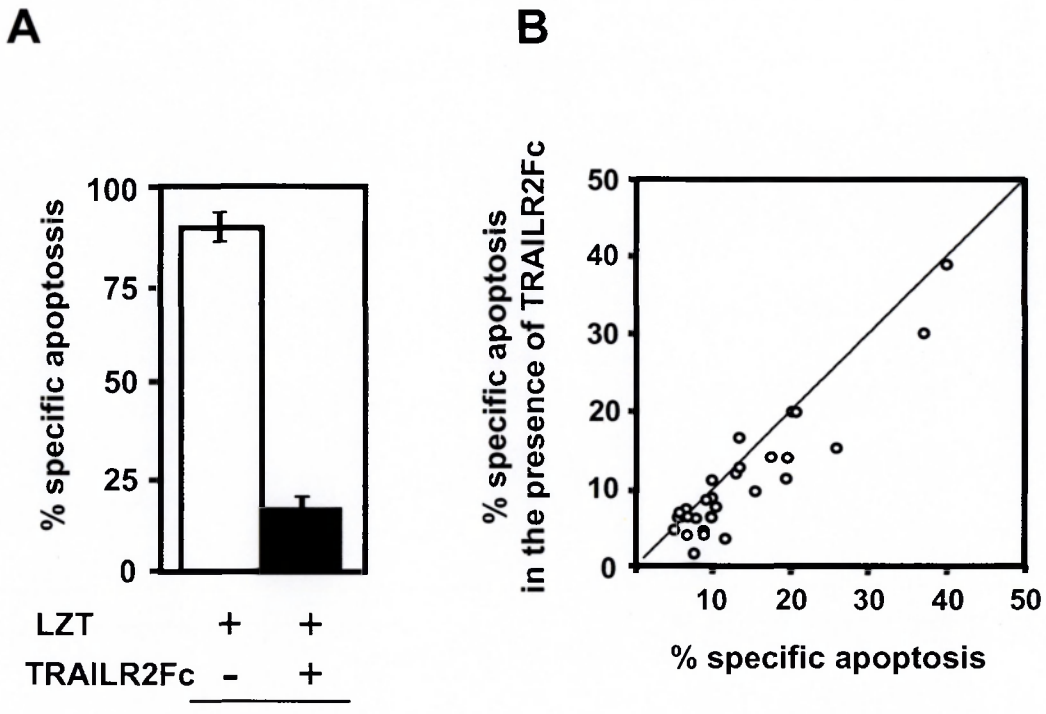

Fig. III.7: αCD4/αCXCR4 induced apoptosis is partially dependent on the TRAIL system. (A) Specific LZT induced apoptosis in Jurkat cells in the presence (black bars) or absence (white bars) of 3 μg/ml TRAIL-R2-Fc. The experiment was performed in triplicates and the results are presented as mean±SD. (B) Specific αCD4/αCXCR4 induced apoptosis, in PBLs from 40 HIV-1 infected individuals, in the presence (Y axis) or absence (X axis) of 30 μg/ml TRAIL-R2-Fc.

As shown in figure III.7 LZT induced apoptosis was almost completely inhibited by TRAIL-R2-Fc. αCD4/αCXCR4 induced apoptosis in PBLs was only partly inhibited by TRAIL-R2-Fc. This partial inhibition was statistically significant ($P<0.001$) in the overall patient group and showed high variability. More specifically TRAIL-R2-Fc inhibited apoptosis in 16 out of 40 patients examined, with percentages of inhibition ranging from 10.7 to 79.8 %.

2.1.3.1 Apoptosis inhibition by TRAIL-R2-Fc correlates with the patient viral load

Inhibition of αCD4/αCXCR4 induced apoptosis by TRAIL-R2-Fc was restricted to some patients and within this patient subgroup levels of inhibition were highly variable. Possible correlations between the clinical and immunological features of the patients and apoptosis inhibition were examined. The effect of CD4 counts, viral load and therapy on the inhibition of CD4/CXCR4 mediated apoptosis was investigated. A statistically significant correlation ($P<0.05$) was found between high levels of inhibition via TRAIL-R2-Fc and high viral load (>5000 copies/ml) (figure III.8).

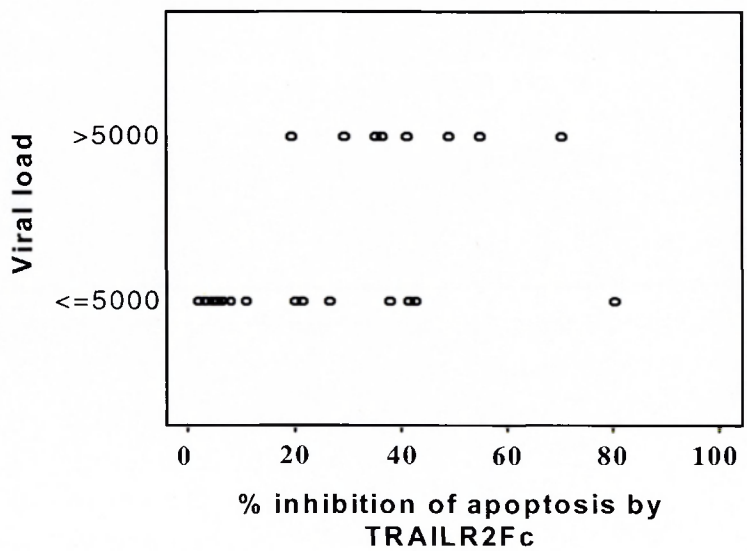

Fig. III.8: Apoptosis inhibition by TRAIL-R2-Fc correlates with the patient viral load.

2.2 Role of caspases in αCD4/αCXCR4 induced apoptosis

2.2.1 The pancaspase inhibitor BD-fmk partly inhibits αCD4/αCXCR4 induced apoptosis

In order to investigate the role of caspases in αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected patients the caspase inhibitor BD-fmk was used. The peptide BD-fmk competes with the natural substrates of all, so far known, caspases for the caspase binding site. The fluoromethylketone(fmk) group binds irreversibly to the active centre of the caspases, thus inhibiting the processing of the natural substrates. For these experiments PBLs from the same patient subgroup as for the experiments with the CD95, TNF, and TRAIL systems were used (table III.3).

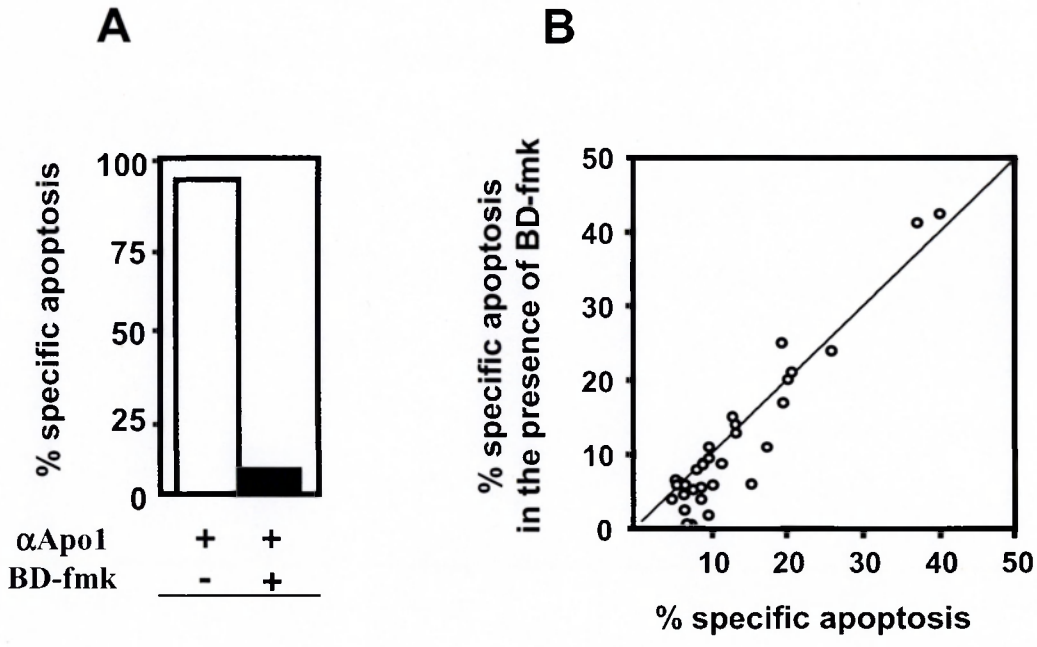

Fig. III.9: αCD4/αCXCR4 induced apoptosis is partly dependent on caspases. (A) Specific αApo1 induced apoptosis in H9 cells in the presence (black bars) or absence (white bars) of 20 μM BD-fmk. The experiment was performed in triplicates and the results are presented as mean±SD. (B) Specific αCD4/αCXCR4 induced apoptosis, in PBLs from 40 HIV-1 infected individuals, in the presence (Y axis) or absence (X axis) of 50 μM BD-fmk.

αCD4/αCXCR4 apoptosis was induced as previously described (see II.2.3.9) but the samples were additionally preincubated in the presence or absence of BD-fmk. As a control for the functionality of BD-fmk, H9 cells were also preincubated in the same manner followed by addition of 1μg/ml αApo1. Apoptosis of H9 was measured after 12 h while apoptosis of PBLs was measured afer 18 h by FSC/SSC analysis. In the H9 cells, αApo1 induced apoptosis was entirely blocked by BD-fmk .

αCD4/αCXCR4 induced apoptosis was partly inhibited by BD-fmk. This partial inhibition was statistically significant ($P<0.01$) but showed great variability within the patient group. More specifically, BD-fmk inhibited CD4/CXCR4 mediated apoptosis in 15 patients with percentages of inhibition ranging from 11.9 to 93 %. These data suggested that in certain patients αCD4/αCXCR4 induced apoptosis is partly mediated by a caspase dependent pathway. Higher concentrations of BD-fmk (up to 100 μM) gave similar results.

2.2.2 Lack of Caspase-8 cleavage during CD4/CXCR4 mediated apoptosis

In the majority of the HIV-1 infected individuals examined αCD4/αCXCR4 induced apoptosis in PBLs was caspase independent . In order to verify this finding, caspase 8 processing was tested by immunoblotting in PBL lysates from some representative patients where no inhibition of apoptosis via BD-fmk was seen.

Caspase-8 is the first molecule of the caspase cascade, providing a molecular link between the Death Inducing Signaling Complex (DISC) and the downstream caspases. Formation of the DISC comprising the adaptor molecule FADD and caspase-8 results in the release of active caspase-8 at the DISC and cleavage of various intracellular death substrates.

$1x10^6$ PBLs from HIV-1 infected individuals were incubated in the presence or absence of αCD4+αCXCR4 and apoptosis was induced as previously described. Cell lysates were prepared (see II.2.5.1) at various time points following stimulation to exclude lack of detection of the active subunit of caspase 8 due to degradation. 50 μg of protein from each sample were loaded on a 12% gel. For the detection of caspase 8 the anti-caspase 8 antibody C-15 was used. As a control Jurkat cells were incubated for 6 h in the presence or absence of 1μg/ml αApo1.

In accordance with the data obtained with the BD-fmk inhibitor, no cleavage of caspase 8 to its active subunits (p43, p41 and p18) was seen during αCD4/αCXCR4 induced apoptosis of PBLs from HIV-infected patients at the various time points indicated (figure III.10). The active p18 subunit was, however, readily detected after stimulation of the Jurkat cells with αApo1.

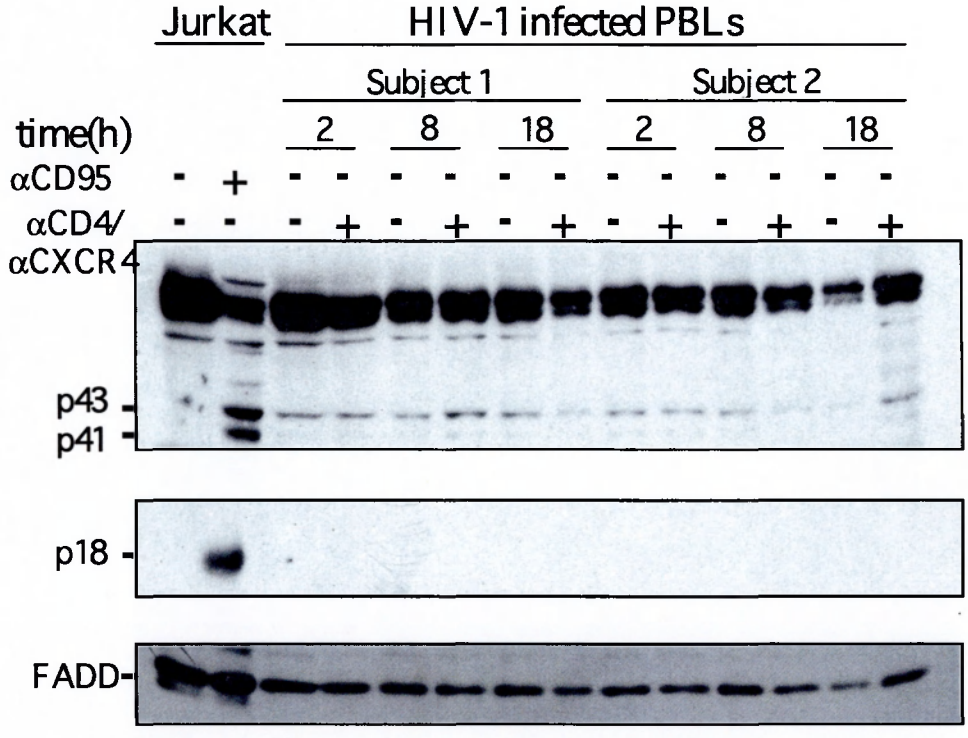

Fig. III.10: Lack of caspase 8 activation during αCD4/αCXCR4 induced apoptosis. Western blot analysis of Caspase 8 in cellular lysates of 10^6 cells. PBLs from HIV-1 positive individuals were either left untreated (-) or treated (+) with αCD4/αCXCR4 and proteolytic processing of caspase 8 was determined at the indicated time points. As a positive control Jurkat cells were either left untreated or treated for 5 hours with 1μg/ml αApo1. The positions of the active fragments p43, p41 and p18 of caspase 8 are indicated. Western blot analysis of FADD is shown at the lower pannel as a loading control.

2.2.3 Lack of PARP cleavage during CD4/CXCR4 mediated apoptosis

PARP is one of the earliest target substrates of Caspase-3 like caspases. Cleavage of PARP into an 85 and 31 kD fragment has been described in all apoptotic processes mediated by caspases.

Processing of PARP was also investigated during αCD4/αCXCR4 induced apoptosis of PBLs from HIV-1 infected individuals by western blot analysis as described for Caspase-8. For PARP detection the anti-PARP antibody CII10 was used.

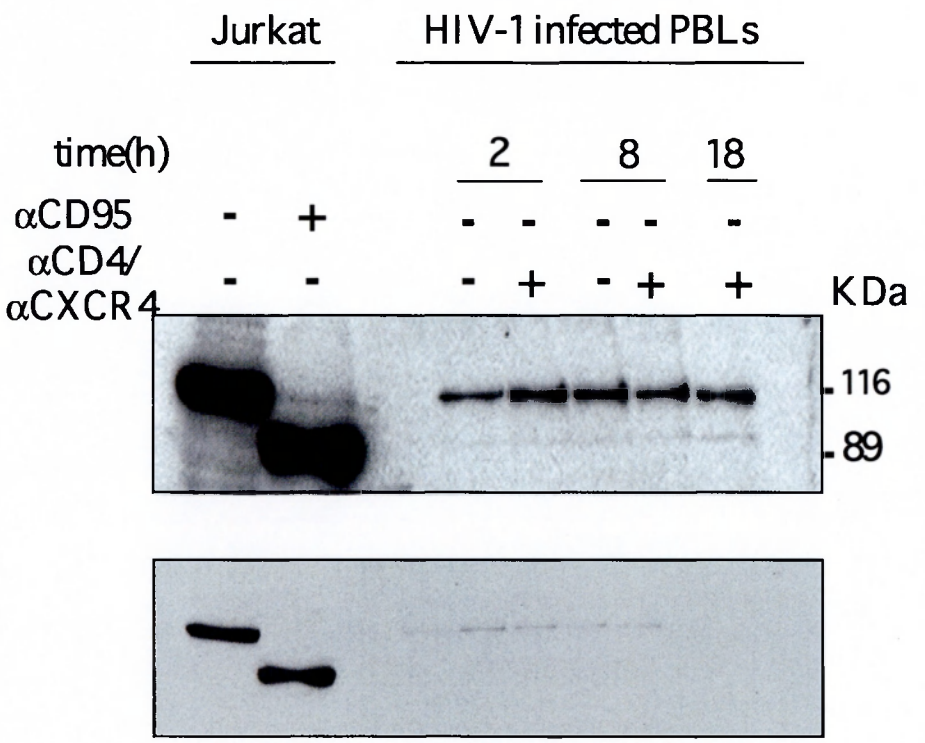

Fig. III.11: Lack of PARP cleavage during αCD4/αCXCR4 induced apoptosis. Western blot analysis of PARP cleavage. PBLs and Jurkat cells were treated as in fig. III.10 and PARP cleavage was determined after the indicated time points. PARP and its cleavage product are indicated. A shorter exposure of the blot is shown in the lower pannel to compensate for the different amounts of protein loaded.

PARP clevage was not observed during αCD4/αCXCR4 induced apoptosis at the various time points indicated (figure III.11) while the p85 cleavage product was readily detected in the control Jurkat cells. Due to unequal loading between the Jurkat cells and the HIV-1$^+$ PBLs a shorter exposure of the blot is also presented.

2.3 Lack of DNA fragmentation during CD4/CXCR4 mediated apoptosis

DNA fragmentation is one of the characteristic features of "classical" apoptosis. However, DNA fragmentation was not observed during the "novel" type of caspase independent cell death. In order to investigate whether DNA is degraded during αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected patients the Nicoletti method was used (see II.2.3.4).

Apoptosis via CD4 and CXCR4 was induced as previously described and assessed after 18 h. The cells were subsequently lysed overnight in a Triton-X-100/PI containing buffer. As a control, PBLs from a healthy individual were incubated for 12h in the presence or absence of 1μg/ml αApo1. The cells were previously cultured for 24 h in the presence of PHA and IL-2. PHA was then washed away and the cells were cultured for 5 days in IL-2 medium in order to increase sensitivity towards αApo1 induced apoptosis.

As shown in figure III.12 induction of apoptosis by αApo1 in PBLs from a healthy individual lead to degradation of the cellular DNA (panel E). In contrast, no subG1 DNA content could be detected in αCD4 or αCXCR4 stimulated PBLs from a HIV-1 infected individual (panels B, C).

In order to ensure that apoptosis was induced via CD4 and CXCR4 an aliquot of the samples was taken prior to lysis and apoptosis induction was evaluated via FSC/SSC analysis. The percentages of dead cells were: 18% in unstimulated PBLs, 31.4% in αCD4 stimulated and 35% in αCXCR4 stimulated cells. The αApo1 treated PBLs from the healthy control showed 85.8% apoptosis. In these samples, the percentage of cell nuclei with fragmented DNA, measured via the Nicoletti assay, was lower than the percentage of dead cells, measured via FSC/SSC analysis (74.4% versus 85.8%). This is due to the fact that nuclei with fragmented DNA are only transiently measurable.

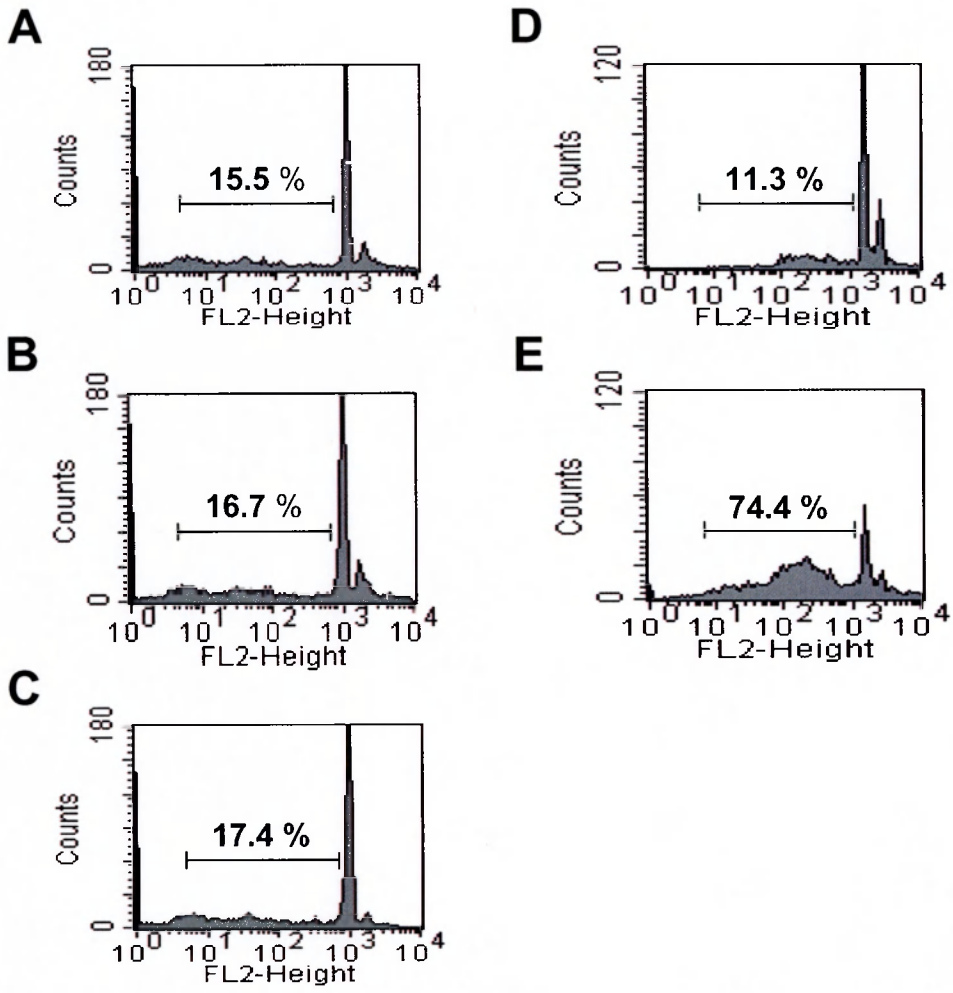

Fig. III. 12: Lack of DNA fragmentation during CD4/CXCR4 mediated apoptosis. The DNA content of unstimulated (A), αCD4 stimulated (B) or αCXCR4 stimulated (C) PBLs from a HIV-1 infected individual and unstimulated (D), or αApo1 stimulated (E) PBLs from a healthy individual, was assessed via flow cytometry. The percentages of nuclei with fragmented DNA are given.

Therefore, DNA degradation in HIV-1$^+$ PBLs was assessed at different time points after stimulation via CD4 and CXCR4. The obtained results were similar to those presented in figure III.12.

2.4 Involvement of mitochondria in αCD4/αCXCR4 induced apoptosis

Apoptosis is accompanied by signs of mitochondrial membrane permeabilization (MMP), including loss of the inner mitochondrial transmembrane potential ($\Delta\Psi m$) and the release of soluble intermembrane proteins via the outer mitochondrial membrane (Kroemer and Reed, 2000; Gross et al., 1999).

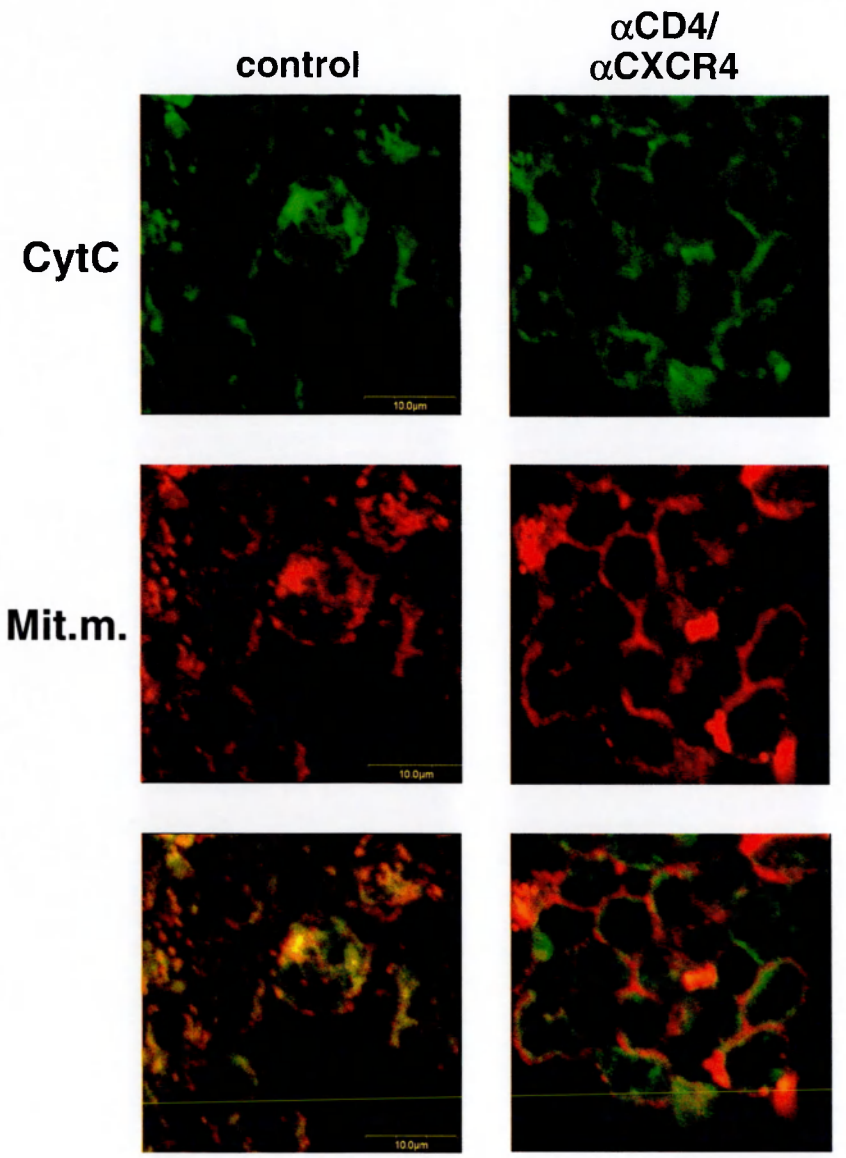

Fig. III.13: Cytochrome c is released during αCD4/αCXCR4 induced apoptosis. Cytochrome c (green fluorescence) is confined to the mitochondria (red fluorescence) in unstimulated PBLs (left panels) while it translocates to the cytosol in αCD4/αCXCR4 stimulated PBLs (right panels). A representative experiment of three is shown.

MMP results in the translocation of cytohrome c (Cyt-c) from the mitochondrial intermembrane space to the cytosol and subsequent cell death (Green and Reed, 1998). In order to investigate the role of mitochondria in αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals, cytochrome c release was assessed by immunofluorescence staining and confocal microscopy.

Apoptosis via CD4/CXCR4 was induced as previously described and cytochrome c release was assessed 12 h after stimulation. As shown in figure III.13 in unstimulated PBLs (control) Cyt-c is confined to the mitochondria as seen by the distinct staining pattern and the colocalisation of the mitochondrial marker and Cyt-c fluorescence signal (lower left panel). Upon stimulation with αCD4/αCXCR4 a more diffuse staining pattern and no colocalisation of the fluorescence signals could be seen (lower right panel). This corresponded to cytochrome c being released in the cytosol.

2.5 Overview of the characteristics of αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals

In this chapter the features of αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals were investigated. Apoptosis mediated via CD4 and CXCR4 was found to be independent of the CD95 and TNF system and no DNA degradation was observed. The dying cells exhibited the characteristic mophological changes of apoptotic cells as evaluated via FSC/SSC analysis. Although due to technical limitations the reduction of the Ψm could not be measured in the patient samples, cytochrome C was found to be released during apoptosis. This showed that mitochondria have a potential role in αCD4/αCXCR4 induced apoptosis. These findings were in accordance with the data previously obtained in cell lines.

However, αCD4/αCXCR4 induced apoptosis in HIV-1$^+$ PBLs showed slower kinetics than in cell lines. It was found to be partly dependent on the TRAIL system. Moreover it was partly inhibited by BD-fmk thus showing a partial dependence on caspases. Nevertheless, in the majority of the patients tested αCD4/αCXCR4 induced apoptosis was independent of caspases as no caspase 8 and PARP cleavage were observed.

Table III.4 gives an overview of the characteristics of αCD4/αCXCR4 induced apoptosis in HIV-1$^+$ PBLs in comparison to "classical" αCD95 induced apptosis and to the "novel" type of αCD4/αCXCR4 induced apoptosis found in cell lines.

Characteristics	αCD95-induced apoptosis	αCD4/αCXCR4-induced apoptosis in cell lines	αCD4/αCXCR4-induced apoptosis in HIV-1$^+$ PBLs
Kinetics	~12 h	~2 h	~18 h
DNA degradation	+	-	-
Cytoplasmic condensation	+	+	+
FSC/SSC changes	+	+	+
Early loss of membrane assymetry	+	+	ND
Reduction of the Ψm	+	+	ND
Cytochrome C release	+	+	+
Caspase-8 cleavage	+	-	-
PARP cleavage	+	-	-
Inhibition via BD-fmk	+	-	partial

Table III.4 : Characteristics of αCD4/αCXCR4 induced apoptosis in HIV-1$^+$ PBLs in comparison to cell lines and to αCD95 induced apoptosis. (ND) not determined due to technical limitations because of fixation of HIV-1$^+$ PBLs.

3 Sensitisation of PBLs from healthy individuals towards αCD4/αCXCR4 induced apoptosis via activation

In previous studies, PBLs from healthy individuals were also found to undergo caspase independent apoptosis upon stimulation via CD4 and CXCR4 (Berndt et al., 1998). However, these findings appear to be contradictory to the data presented in chapter 1. The experiments presented here were designed in order to investigate whether the activation state of the PBLs determines sensitivity versus resistance towards αCD4/αCXCR4 induced apoptosis. In order to gain more insight into the potential role of αCD4/αCXCR4 induced apoptosis the sensitivity of different PBL subpopulations was also assessed.

3.1 Mitogenic stimulation renders PBLs sensitive to αCD4/αCXCR4 induced apoptosis

PBLs were isolated from whole blood of healthy individuals as described in materials and methods (II.2.2.1). The cells were subsequently cultured for 12 h in RPMI medium containing 10 ng/ml of the mitogen PHA-P. PHA-P was then washed away and the cells were kept in RPMI medium supplemented with 25 U/ml IL-2. 10^5 PBLs either freshly isolated or PHA-P/IL-2 stimulated were incubated in the presence or absence of αCD4 or αCXCR4.

Apoptosis was induced as previously described (see II.2.3.9) and measured via FSC/SSC analysis 2 h following stimulation. As shown in figure III.14 freshly isolated PBLs were not sensitive to αCD4 or αCXCR4 induced apoptosis. PBLs stimulated via PHA-P/IL-2 for 24 or 48 h showed sensitivity to apoptosis mediated via CD4 and CXCR4. Sensitivity declined in PBLs kept in IL-2 medium for 72h and high levels of spontaneous apoptosis were usually observed in these samples.

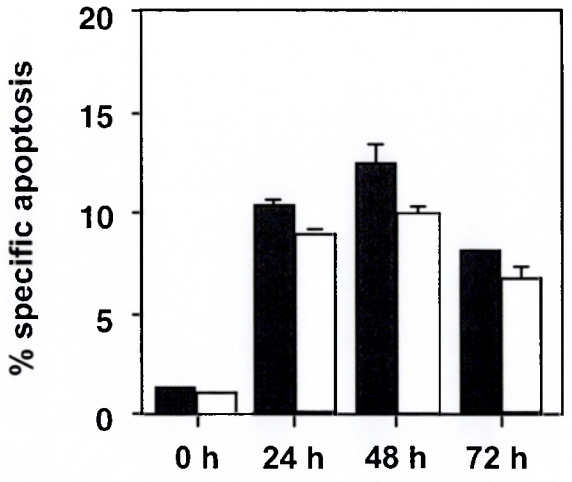

Fig. III.14: Mitogenic stimulation renders PBLs sensitive to αCD4/αCXCR4 induced apoptosis. PBLs either freshly isolated or stimulated with PHA-P/IL-2 for 24, 48 or 72 h were incubated with 10 µg/ml αCD4 (grey bars) or αCXCR4 (white bars) and a crosslinking secondary antibody. Specific apoptosis was calculated as described in Materials and Methods. One representative experiment of 10 performed in triplicates is shown.

3.2 $CD4^+$ activated PBLs are sensitive to αCD4 and αCXCR4 induced apoptosis

3.2.1 Immunomagnetic separation of PBL subpopulations

CD4, CD8, CD45RA and CD45RO negative PBL subpopulations were obtained as described in II.2.2.2. $5x10^7$ PBLs previously incubated in IL-2 containing medium for 24 h were incubated with 25 µg/ml of a monoclonal antibody against either CD4 (IOT4) or CD8 (OKT8) in order to obtain CD4 and CD8 negative populations respectively. For the CD45RA and RO negative subpopulations the HI100 and UCHL1 antibodies were used. Additionally, an antibody against CD16 (3G8) and one against CD19 (HD37) were included in order to deplete the CD16 expressing NK cells and granulocytes and the CD19 expressing B cells.

The separation process here described is based on «negative selection» of PBL subpopulations where the non-desired fraction is depleted by binding of the appropriate antibody to the magnetic beads while the remaining fraction is the one of interest. For

simplicity the obtained populations were designated as $CD4^+$, $CD8^+$, $CD45RA^+$ and $CD45RO^+$ (corresponding to $CD8^-$, $CD4^-$, $CD45RO^-$ and $CD45RA^-$ cells respectively).

3.2.2 Characterisation of the PBL subpopulations via surface staining

3.2.2.1 Characterisation of $CD4^+$ and $CD8^+$ PBL subpopulations

In order to assess the purity of the subpopulations obtained via immunomagnetic separation the surface expression of the antigen of interest was evaluated by immunofluorescence staining and flow cytometry (see II.2.2.3.1). The CD4 and CD8 receptors were detected by using FITC-conjugated antibodies against CD4 (HP2/6) or CD8 (OKT8) (provided by Dr. G. Moldenhauer, German Cancer Research Centre).
The CD4 and CD8 expression levels of the PBL subpopulations as well as of unseparated PBLs are shown in table III.5. The $CD4^+$ and $CD8^+$ subpopulations were ≥ 95 % pure.

	αCD4		αCD8	
Population	[%]	MFI	[%]	MFI
PBL	64.5	38.2	22.4	147.6
$CD4^+$	**96.4**	33.1	4.5	29.3
$CD8^+$	3.8	14.6	**97.9**	173.3

Table III.4 : CD4 and CD8 expression in unseparated PBLs and PBL subsets. The numbers indicate percentages of cells that interact with the monoclonal antibodies [%] and mean fluorescent intensity (MFI) acquired by flow cytometry. The values indicated in the table are representative of triplicates.

3.2.2.2 Characterisation of CD45RA$^+$ and CD45RO$^+$ PBL subpopulations

The surface expression of the antigens of interest was assessed by immunofluorescence staining and flow cytometry. The HI100 and UCHL1 antibodies were used for the detection of CD45RA and CD45RO respectively, followed by a PE-conjugated secondary antibody. As a control, cells were incubated with the secondary antibody alone. As shown in table III.5 the subpopulations obtained via immunomagnetic separation were > 95 % pure.

The CD45RA and RO markers were used in order to define naive versus activated PBLs. In order to further characterise the naive or activated phenotype of the obtained subsets the expression levels of the activation markers CD25, CD69 and CD95 were examined. CD45RO$^+$ cells expressed high levels of all three markers in contrast to CD45RA$^+$ cells.

	CD45RA$^+$		**CD45RO$^+$**	
	[%]	MFI	[%]	MFI
control	5.4	14.9	1.46	11.4
CD45RA	**97.8**	103.1	5.8	41.5
CD45R0	6.7	34.8	**96.7**	93.1
CD3	97.3	92.8	98.9	107
CD8	31.2	159.5	9.2	65.3
CD25	9.4	20.7	69.7	31.9
CD69	4.1	21.3	73.8	46.2
CD95	8.8	17	95.8	32.2

Table III.5 : Phenotypic characterisation of CD45RA$^+$ and CD45RO$^+$ PBL subsets. The numbers indicate percentages of cells that interact with the monoclonal antibodies. [%], and mean flrorescent intensity (MFI) acquired by flow cytometry. The values indicated in the table are representative of triplicates.

Subsequently, CD4 and CXCR4 expression levels were assessed in the $CD45RA^-$ and $CD45RO^-$ subpopulations in comparison to unseparated PBLs. For the detection of CXCR4 the 12G5 antibody was used. As shown in table III.6 the proportion of CD4 expressing cells in the $CD45RA^+$ population was approximately 50% less than in the $CD45RO^+$ population (46.2 % versus 81.7 %). CXCR4 was also expressed at a 3 fold higher level in the $CD45RO^+$ subpopulation (54.5 % versus 16.5 %).

	αCD4		αCXCR4	
Population	[%]	MFI	[%]	MFI
PBL	65.8	38.2	32.5	20.8
$CD45RA^+$	46.2	34.7	16.5	19
$CD45RO^+$	81.7	36.6	54.5	24

Table III.6 : CD4 and CXCR4 expression in PBLs and $CD45RA^+$, $CD45RO^+$ subpopulations. The numbers indicate percentages of cells that interact with the monoclonal antibodies [%] and mean fluorescent intensity (MFI) acquired by flow cytometry. The values indicated in the table are representative of triplicates.

3.2.3 Sensitivity of PBL subpopulations to αCD4 and αCXCR4 induced apoptosis

3.2.3.1 $CD4^+$ lymphocytes are sensitive to αCD4 and αCXCR4 induced apoptosis

The sensitivity of the $CD4^+$ and $CD8^+$ PBL subpopulations to αCD4 and αCXCR4 induced apoptosis was examined. Apoptosis via CD4 and CXCR4 was induced as previously described and measured using the PI exclusion assay and flow cytometry. In accordance to what has been previously reported only $CD4^+$ cells were sensitive to apoptosis mediated via CD4 and CXCR4 (figure III.15) (Berndt et al., 1998).

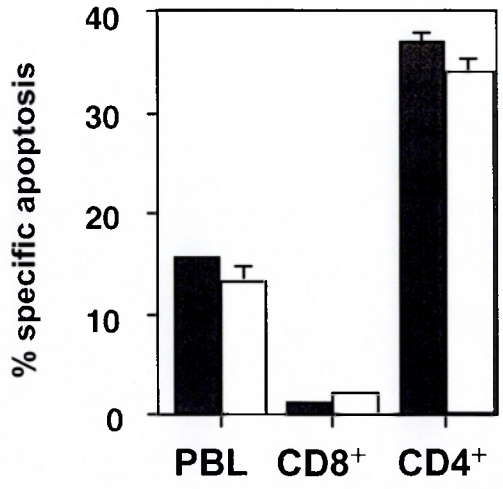

Figure III.15 : CD4$^+$ lymphocytes are sensitive to CXCR4 and CD4 mediated apoptosis. PBLs or CD4$^+$, CD8$^+$ PBL subsets were incubated with 10 µg/ml αCD4 (grey bars) or αCXCR4 (white bars) and a crosslinking secondary antibody. Specific apoptosis was calculated as described in Materials and Methods. One representative experiment of 3 performed in triplicates is shown.

3.2.3.2 CD45RO$^+$ lymphocytes are sensitive to αCD4 and αCXCR4 induced apoptosis

Subsequently, the sensitivity of the CD45RA$^+$ and CD45RO$^+$ PBL subpopulations to αCD4 and αCXCR4 induced apoptosis was examined. Apoptosis via CD4 and CXCR4 was induced as previously described and measured using the PI exclusion assay and flow cytometry. As shown in figure III.16 approximately 30% of the CD45RO$^+$, activated lymphocytes were sensitive to apoptosis mediated via CD4 and CXCR4. In contrast no apoptosis induction was observed in the CD45RA$^+$, naive lymphocytes although they expressed both CD4 and CXCR4 receptors, albeit in lower levels than CD45RO$^+$ cells.

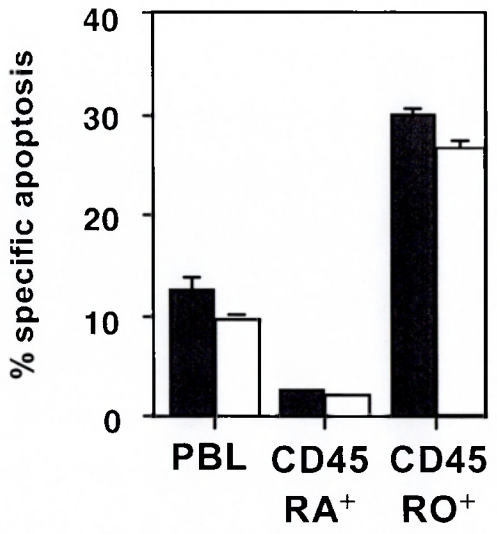

Figure III.16 : CD45RO$^+$ lymphocytes are sensitive to CXCR4 and CD4 mediated apoptosis. PBLs or CD45RA$^+$, CD45RO$^+$ PBL subsets were incubated with 10 µg/ml αCD4 (grey bars) or αCXCR4 (white bars) and a crosslinking secondary antibody. Specific apoptosis was calculated as described in Materials and Methods. One representative experiment of 10 performed in triplicates is shown.

Therefore, as demonstrated by these experiments CD4$^+$ activated lymphocytes are susceptible to αCD4 and αCXCR4 induced «novel» type of apoptosis. These findings highlight the relevance of this type of apoptosis to HIV-1 pathogenesis since this lymphocyte population is the one mainly depleted during infection.

4 Structure-function studies of the CD4 and CXCR4 receptors

In order to gain some initial insight into the mechanism of CD4 and CXCR4 mediated apoptosis structure-function studies of the two receptors were carried out. These aimed to identify structural determinants of the receptors required for the apoptotic signaling and for possible interactions with downstream molecules. For this purpose wild type, chimeric or mutant CXCR4 or CD4 receptors were expressed in the human B-lymphoblastoid cell line BJAB, as this cell line is CD4 and CXCR4 negative and can be stably transfected with high efficiency. Subsequently the sensitivity to apoptosis of cells expressing one of the various chimeric and mutant receptors in comparison to cells expressing one of the wild type receptors was assessed.

4.1 Structure –function analysis of the CXCR4 receptor

The creation of receptor chimeras, hybrid molecules composed of complementary portions of related receptors that have distinguishing biochemical or biological characteristics, is a powerfull approach for the identification of domains that impact activity.

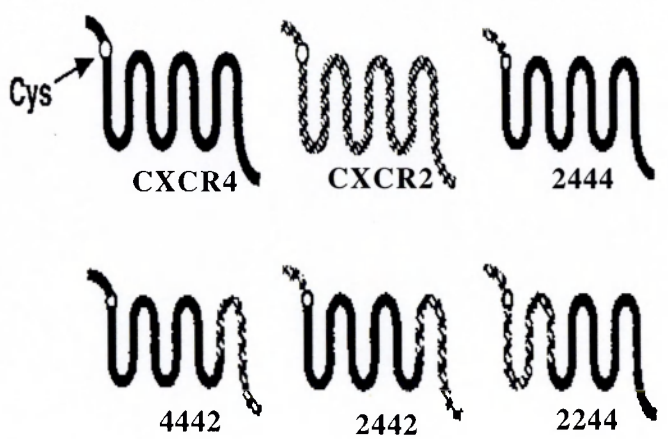

Fig. III.17: CXCR4-CXCR2 chimeras. Chimeric receptors based on CXCR4 and CXCR2 are depicted schematically. Aminoterminal domain exchanges were performed at the conserved cysteine residue at position 28 of CXCR4.
(Figure redrawn from Lu et al., 1997 PNAS)

In order to identify regions of CXCR4 required for apoptosis induction chimeric molecules between the CXCR4 and CXCR2 receptors were used. These receptors share approximately 35% amino acid identity. However, CXCR2 has not been reported to serve as a HIV-1 coreceptor.

The following chimeric receptors, where either single or multiple CXCR4 domains were replaced with corresponding CXCR2 regions, were used: 2444, 4442, 2442, 2244 (the 2444 and 4442 chimeric receptor cDNAs were provided by Dr. S. Peiper, University of Louisville, while the CXCR2 receptor cDNA was provided by Dr. Robert Doms, University of Pensylvania), (figure III.17).

4.1.1 Generation of CXCR4-CXCR2 receptor chimeras and CXCR4 receptor mutants

Chimeric receptors composed of CXCR4 and CXCR2 were constructed by the PCR-ligation-PCR approach. To create chimeras, complementary cDNAs of the two parental receptors were amplified from cDNA templates and the blunt-ended amplification products were ligated after phosphorylation. The product encoding the desired hybrid was then amplified with the appropriate upstream and downstream primers from the respective parental receptors (see II.1.6.3). Products of the predicted size were cloned into the pBS vector, screened and sequenced. The chimeric receptor cDNAs were subsequently cloned into the pcDNA3 vector.

In order to study the role of the C-terminal domain of CXCR4 in the transmission of the apoptotic signal a deletion mutant receptor (CXCR4 Δcyto) was used. CXCR4 was truncated at the amino acid 318 by altering the codon 319 into a stop codon using standard PCR methods (primers shown in II.1.6.3).

4.1.2 Generation and characterisation of stable transfectants

The CXCR4 and CXCR2 negative cell line BJAB was used for the generation of stable transfectants expressing one of the following receptors: CXCR4 wt, CXCR2 wt, 2444, 4442, 2442, 2244, CXCR4 Δcyto, or the pcDNA3 vector alone. The transfection protocol described in II.2.4.9 was followed. Cell surface expression of the molecule of interest was tested via immunofluorescence staining. The CXCR2 , 2444, 2442 and

2244 receptors were detected by using a monoclonal antibody against the amino terminus of the CXCR2 receptor (10G2), while the CXCR4, 4442 and CXCR4 Δcyto receptors were detected by using a monoclonal antibody against the first and second extracellular loops of the CXCR4 receptor (12G5). A ß-Phycoerythrin conjugated secondary antibody was used. The results of the immunofluorescence staining of a representative clone from each transfectant group are shown in table III.7 and in figure III.18.

	Control		αCXCR2		αCXCR4	
Cells	[%]	MFI	[%]	MFI	[%]	MFI
BJAB	0.2	18	5.3	15	7.1	11
HPB-ALL	0.83	7.1	-	-	80	55.4
Vector (pcDNA3) #5	0.29	4.2	1.7	3.15	5.6	8
CXCR4 wt$^+$ #18	0.41	16	-	-	**99.3**	66.9
CXCR2$^+$ #12	0.42	8.5	**98**	162.5	-	-
CXCR4 Δcyto$^+$ #11	0.8	25	-	-	**99.5**	773.8
2444$^+$ #12	5.9	12.6	**98.7**	156.6	-	-
4442$^+$ #13	1.7	13.45	-	-	**98.8**	157.1
2442$^+$ #12	0.29	14	**99.3**	401.3	-	-
2244$^+$ #5	0.15	3.9	**85.7**	27.7	-	-

Table III.7 : Surface expression of wild type, mutant and chimeric CXCR4 receptors in stable transfectants compared to untransfected BJAB and HPBALL cells. The numbers indicate percentages of cells that interact with the monoclonal antibodies [%] and mean fluorescent intensity (MFI) acquired by flow cytometry. The values indicated in the table are representative of triplicates.

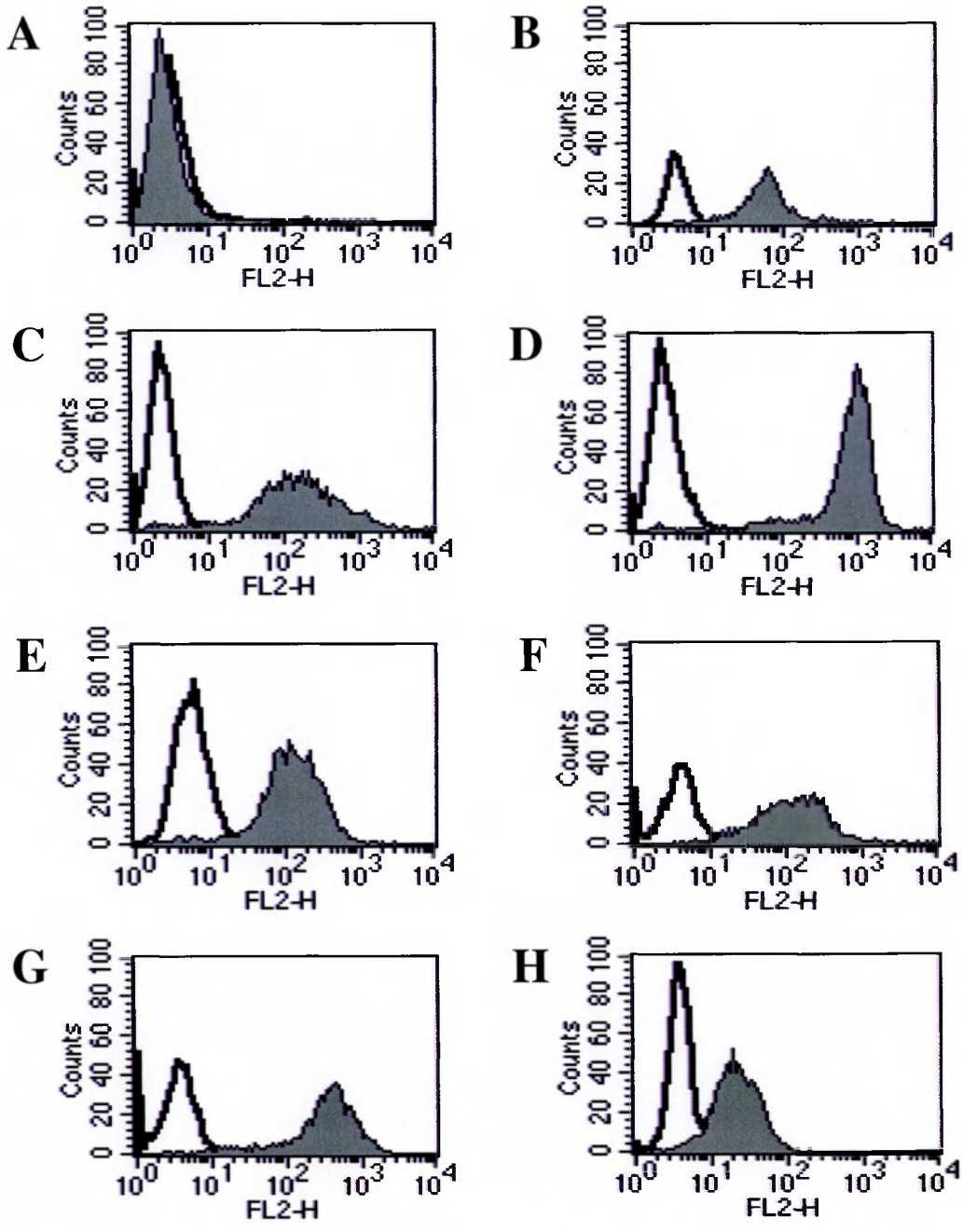

Fig. III.18 : Cell surface expression of wt, mutant and chimeric CXCR4 receptors stably expressed in BJAB cells. Surface expression levels of CXCR4, CXCR2 , mutant and chimeric receptors were evaluated via flow cytometry after staining with the secondary antibody alone (control, white peak) or with an appropriate monoclonal antibody (grey peak), (A) vector #3, (B) CXCR4 #6, (C) CXCR2 #12, (D) CXCR4 Δcyto #11, (E) 2444 #12, (F) 4442 #13, (G) 2442 #12, (H) 2244 #5.

4.1.3 Sensitivity of the transfectants towards αCXCR4 induced apoptosis

The selected transfectants were tested for their sensitivity towards CXCR4 induced apoptosis. CXCR4 mediated apoptosis was induced as previously described (see II.2.3.9). and was evaluated following a 2 h incubation via FSC/SSC analysis. As shown in fig.III.19 the CXCR4 expressing transfectants were sensitive to anti-CXCR4 induced apoptosis. Stimulation of CXCR2 positive transfectants by anti-CXCR2 did not however lead to induction of apoptosis thus validating the choice of this receptor for the construction of the chimeric molecules. Replacement of the amino terminus of CXCR4 up to the conserved cysteine residue at position 28 (chimera 2444) did not affect the induction of apoptosis. The same was seen with the chimera 4442 thus indicating that the third extracellular loop and the C-terminal of the CXCR4 receptor are also dispensable for the apoptotic function. Deletion of the C-terminal domain (mutant CXCR4 Δcyto) had no effect on anti-CXCR4 induced apoptosis, further indicating that this domain is not involved in the apoptotic signaling.

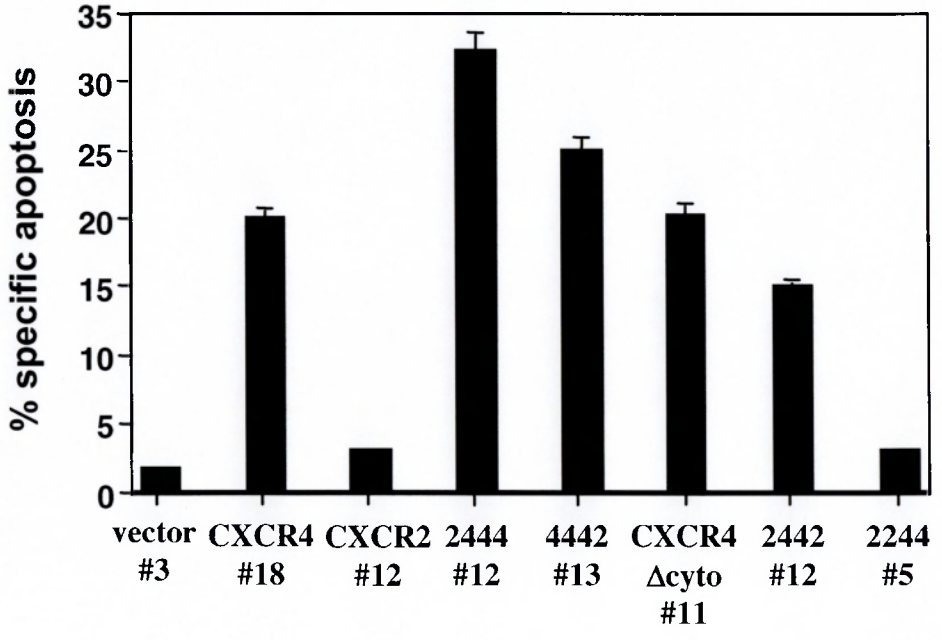

Fig. III.19: Apoptosis induction in cells expressing wild type, mutant or chimeric CXCR4. Apoptosis was assessed by flow cytometry (FSC/SSC analysis). The results shown are representative of ten independent experiments and the values indicate mean ± SD of triplicates.

Since the amino terminal domain and the third extracellular loop of CXCR4 could be individually replaced without ablating transmission of the apoptotic signal it was tested whether replacement of both regions is also tolerated. Chimera 2442 mediated anti-CXCR4 induced apoptosis thus suggesting that the region from the first cysteine residue till the third extracellular loop is sufficient for the apoptotic function. In order to examine the roles of the first intracellular and extracellular loops in the transmission of the apoptotic signal the chimera 2244 was examined. Chimera 2244 did not mediate anti-CXCR4 induced apoptosis. This indicated that the area from the second intracellular loop till the third extracellular loop is critical for the transmission of the apoptotic signal.

4.2 Analysis of CD4 mutant receptors

In order to determine which part of the CD4 receptor plays a role in the transduction of the apoptotic signal stable transfectants were generated expressing either the CD4 wild type receptor or one of a series of intracellularly truncated CD4 receptors bearing a COOH terminal truncation at the indicated amino acid : 431, 429, 419, 411, 398.

Moreover a CD4 mutant receptor was used bearing a mutation at the p56 lck kinase binding site, designated CD4 lck-, which prevents the kinase to associate with the receptor (all CD4 mutant receptor cDNAs were kindly provided by Dr. Waldemar Kolanus, University of Munich) (figure III.20).

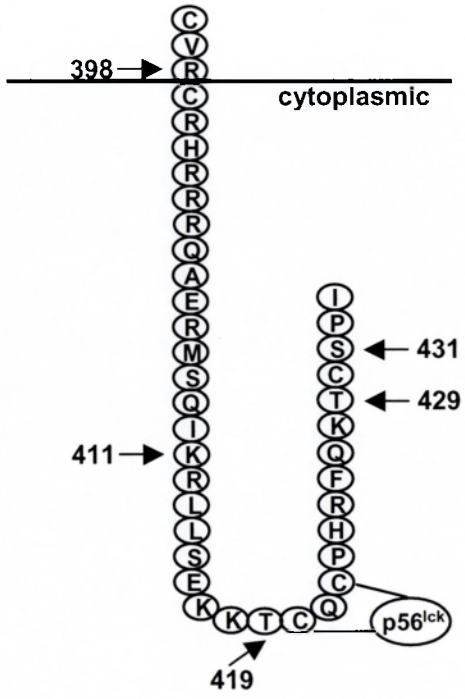

Fig. III.20: CD4 receptor mutants. Schematic representation of the CD4 mutants investigated in this study. Truncations at amino acid residues 431, 429, 419, 411 and 398 partially or totally delete the cytoplasmic tail. The CC- to AA substitution in the dicysteine motif 420 and 422 disrupts CD4-$p56^{lck}$ association.

4.2.1 Generation and characterisation of stable CD4 transfectants

For the generation of stable CD4 transfectants BJAB cells were transfected as described in II.2.4.9 either with the CD4 wild type(wt) receptor or one of the receptor mutants (figure III.20). As a control BJAB cells were transfected with the empty vector pEGFP-N1. Clonal cell populations were expanded and the cell surface expression of the molecule of interest was tested via immunofluorescence staining. The FITC-conjugated anti-CD4 antibody RPA-T4 was used for the detection of the receptors and a FITC-conjugated mouse IgG1 was used as an isotype control.

Cells	Control		αCD4	
	[%]	MFI	[%]	MFI
BJAB	1.44	10	6,2	11,74
HPB-ALL	0.83	5.4	99.9	283.6
Vector (pEGFP-N1) #5	0.5	5.3	1.9	6.5
CD4 wt$^+$ #6	2	12.4	**97.7**	40
CD4 lck– $^+$ #4	0.9	5	**89.5**	32.3
398$^+$ #15	1.9	12	**97.6**	87.2
411$^+$ #18	0.04	10.3	**89.5**	100.2
419$^+$ #6	0.3	8.4	**97.4**	71.7
429$^+$ #5	0.5	6.4	**92.3**	40.9
431$^+$ #17	3	12.2	**92.3**	78.6

Table III.8: Surface expression of wild type and mutant CD4 in stable transfectants compared to untransfected BJAB and HPBALL cells. The numbers indicate percentages of cells that interact with the monoclonal antibodies [%] and mean fluorescent intensity (MFI) acquired by flow cytometry. The values indicated in the table are representative of triplicates.

The CD4 receptor expressing clones selected via surface staining were subsequently expanded and the surface staining was repeated at various time intervals in order to determine the stability of expression. According to the surface expression of the molecule of interest two to three clones were finally selected. The results of the immunofluorescence staining of a representative clone from each receptor transfectant are shown in table III.8 and figure III.21.

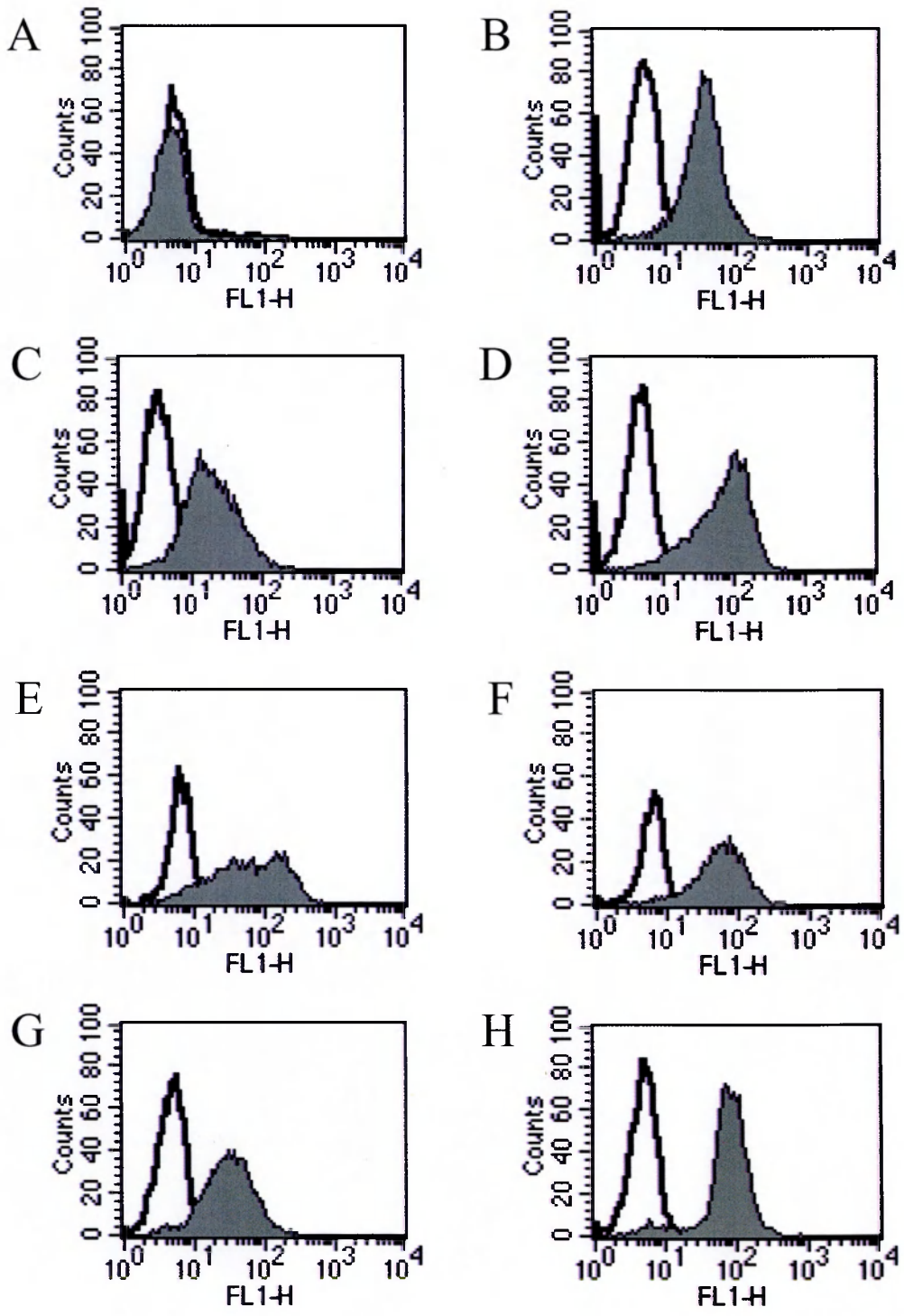

Fig. III.21 :Cell surface expression of CD4 wt or mutants stably expressed in BJAB cells. Surface levels of CD4 were evaluated via flow cytometry after staining with an isotype control (white peak) or an αCD4 (grey peak) antibody, (A) vector #5, (B) CD4 wt #6, (C) lck- #4, (D) 398 #15, (E) 411 #18, (F) 419 #6, (G) 429 #5, (H) 431 #6.

4.2.2 Sensitivity of the transfectants towards αCD4 induced apoptosis

The selected transfectants stably expressing the CD4 wt or mutant receptors were then assessed for their sensitivity towards CD4 mediated apoptosis. CD4 mediated apoptosis was induced as previously described (see II.2.3.9) and was measured after a 2 h incubation via FSC/SSC analysis. Cells expressing receptors truncated at residue 431 and 429 underwent apoptosis like those expressing the wild-type CD4 (figure III.22). Similar levels of apoptosis induction were also observed in cells expressing a mutant receptor that does not associate with $p56^{lck}$. This demonstrated that the apoptotic signal is independent of $p56^{lck}$ signaling activity. This was further confirmed with the CD4 construct truncated at residue 419 as cells expressing this construct also underwent apoptosis. Moreover, apoptosis was induced via the mutant 411 receptor at levels comparable to the wild type receptor. Surprisingly, apoptosis could still be induced, albeit at significantly lower levels, in cells expressing the 398 mutant where the cytoplasmic tail is entirely truncated.

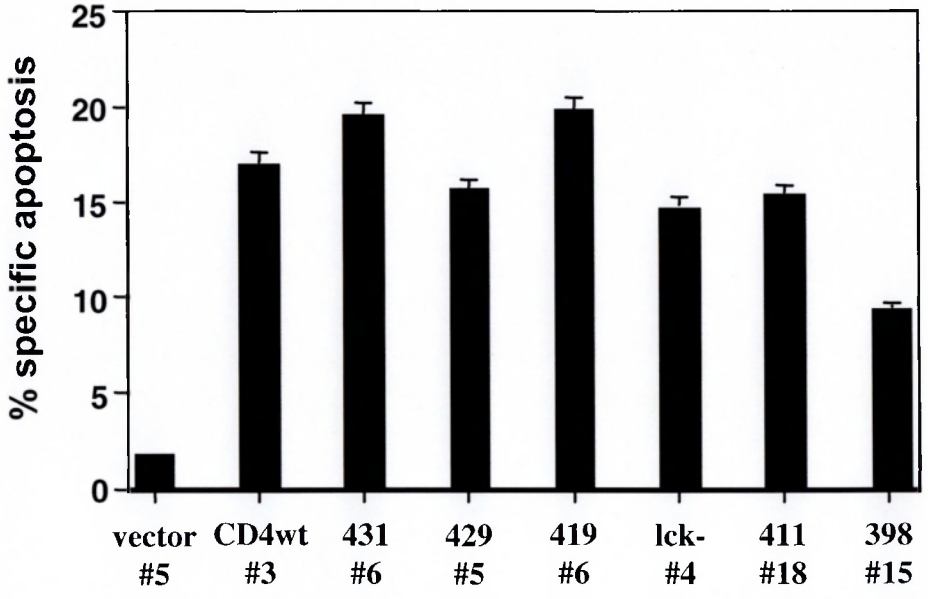

Fig. III.22 : Apoptosis induction in cells expressing wild type or mutant CD4. Apoptosis was assessed by flow cytometry (FSC/SSC analysis). The results shown are representative of ten independent experiments and the values indicate mean ± SD of triplicates.

5 Investigation of the mechanism of αCD4 induced apoptosis in a T cell line system

Preliminary studies on the mechanism of the novel type of CD4 and CXCR4 mediated cell death, in HPBALL cells, showed that the molecules normally associated with signaling by these receptors, $p56^{lck}$ and $G_i\alpha$ respectively, were not involved in the apoptotic pathway (Berndt et al., 1998). In order to gain more insight into the signaling mechanism of this novel type of cell death the experiments were focused on αCD4 induced apoptosis as the CD4 receptor provides a simpler experimental system compared to the less well characterised and structurally more complex CXCR4 receptor. The potential recruitment of signaling molecules to the CD4 receptor complex as well as the role of tyrosine phosphorylation in the apoptotic signaling were investigated.

5.1 Optimization of CD4 immunoprecipitation

In order to study the association of signaling molecules the CD4 receptor was immunoprecipitated as described in II.2.5.5. To optimize the immunoprecipitation conditions different detergent subtances were tested for their ability to solubilise the CD4 receptor while retaining complex formation with signaling molecules.

For this purpose 10^7 unstimulated HPBALL cells were lysed as described in II.2.5.1 using a lysis buffer containing either Triton X-100 (see II.1.2) or Brij58 (see II.1.2) or Digitonin (10 mM triethanolamine pH 8, 150 mM NaCl, 1mM EDTA, 10% glycerol, digitonin 2% (v/v), 10 µM Na-orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin) . Subsequently the CD4 receptor was immunoprecipitated as described in 2.5.5 using 2 µg of the HP2/6 anti-CD4 antibody. The samples were then analysed by western bloting using an antibody against $p56^{lck}$ (1.5.1) as this kinase is constantly associated to the CD4 receptor. As shown in figure III.23 $p56^{lck}$ association was readily detected in Triton X-100 lysed samples even at low concentrations of detergent (lanes A,B,C). A weaker signal was detected in Brij58 treated samples (lane F) while much lower amounts of $p56^{lck}$ were detected in digitonin treated samples (lanes D, E).

Therefore, for further experiments a 1% Triton X-100 containing lysis buffer was used (II.1.2).

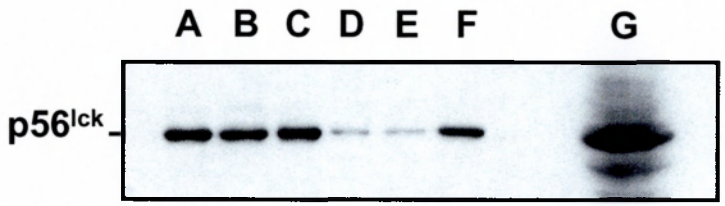

Fig. III.23: Optimization of CD4 immunoprecipitation. p56 lck expression in unstimulated HPBALL cells lysed in a buffer containing either Triton X-100 (A-C) at various concentrations 1% (A), 0.5 % (B), 0.1 % (C), or digitonin at 2%(D) and 1 %(E), or Brij58 1% (F) and immunoprecipitated using an αCD4 antibody. (G) $p56^{lck}$ expression in HPBALL whole cell lysate.

5.2 Lack of recruitment of signaling molecules to the CD4 receptor complex during apoptosis

In order to investigate the recruitment of signaling molecules to the CD4 receptor upon induction of apoptosis HPBALL cells were metabollically labelled as described in II.2.5.4. Apoptosis was then induced by incubating $2x10^7$ cells per sample in the absence or presence of αCD4 and then tranferring them to previously coated 12 well plates (see II.2.3.9) at $2x10^6$ cells per well for the indicated time points (fig. III.24). The cells were then collected and CD4 was immunoprecipitated in unstimulated and stimulated cells as described in II.2.5.5. The samples were then analysed by one dimensional gel electrophoresis (see II.2.5.4).

As shown in figure III.24 no differences in the CD4 receptor complex composition were seen between unstimulated (lanes A,B) or CD4 stimulated cells (C-H) at the various time points tested after a seven day exposure of the film. Similar results were obtained after a twenty one day exposure of the film. This finding suggests lack of recruitment of signaling molecules to the CD4 receptor complex during apoptosis induction.

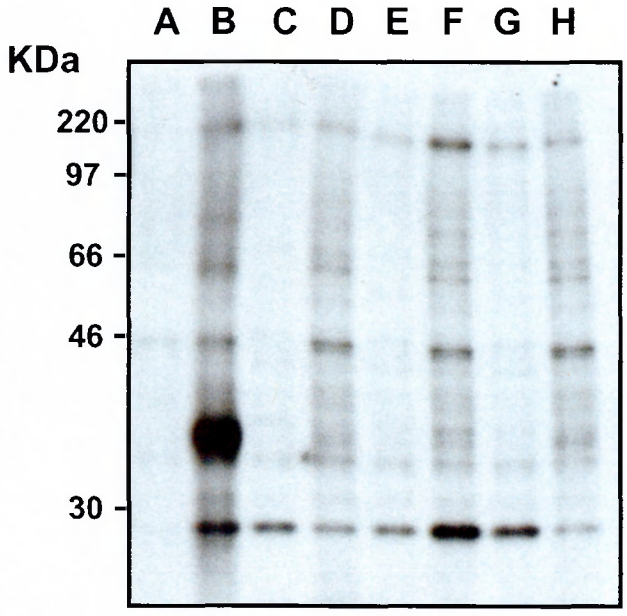

Fig. III.24: Lack of recruitment of signaling molecules to the CD4 receptor complex during apoptosis. Unstimulated (A, B) or CD4 stimulated for 5 min (C, D), 10 min (E, F) or 30 min (G, H) ^{35}S labelled HPBALL cells were immunoprecipitated with either protein A IgG coupled control beads (A, C, E, G) or protein G beads/ 2 µg anti-CD4 (HP2/6) (B) or protein G beads alone (D, F, H). A seven day exposure of the film is shown.

5.3 Tyrosine Phosphorylation of signaling molecules is not involved in αCD4 induced apoptosis

Since signaling molecules did not seem to be recruited to the CD4 receptor complex upon apoptosis induction the role of phosphorylation was examined.

5.3.1 Lack of Tyrosine phosphorylation of signaling molecules after induction of CD4 mediated apoptosis

HPBALL cells were incubated in the presence or absence of αCD4 as previously described (II.2.3.9). In order to compare phosphorylation events following an apoptotic or a nonapoptotic stimulus, however, the CD4 antibody was further crosslinked by either coated (apoptotic stimulus) or soluble (nonapoptotic stimulus) secondary

antibody. The samples were incubated in the presence of either coated or soluble crosslinking antibody for the indicated time points (figure III.25).

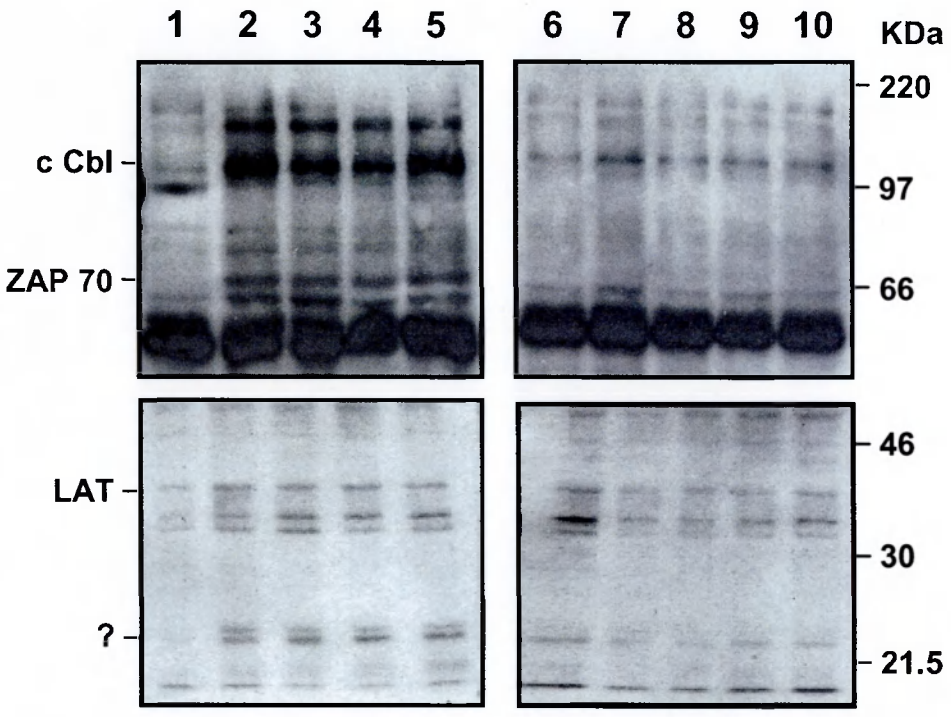

Fig. III. 25 : Lack of Tyrosine phosphorylation of signaling molecules after induction of CD4 mediated apoptosis. Immunoblotting with an anti-phosphotyrosine Ab (PY99) of 10^6 HPBALL cells either left untreated (lanes 1, 6) or stimulated via αCD4+soluble secondary Ab (lanes 2-5) or αCD4+coated secondary Ab (lanes 7-10) for 1 min (lanes 2, 7), 5 min (lanes 3, 8), 10 min (lanes 4, 9) and 30 min (lanes 5, 10). One representative experiment of five is shown.

Cell lysates were subsequently prepared as described in II.2.5.1 and analysed by immunoblotting with an anti-phosphotyrosine antibody.

As shown in figure III.25 stimulation of the CD4 receptor by a nonapoptotic stimulus (lanes 1-5) lead to phosphorylation of the adaptor molecules c-Cbl and LAT, the protein tyrosine kinase ZAP-70 and a nonidentified 23 kDa protein, already 1 min

after stimulation. In contrast no specific phosphorylation events were observed after induction of αCD4 induced apoptosis (lanes 6-10).

5.3.2 PP2 does not inhibit αCD4 induced apoptosis

In order to verify that αCD4 induced apoptosis is independent of tyrosine phosphorylation the tyrosine kinase inhibitor PP2 was used. 10^5 HPBALL cells were incubated for 40 min in the absence or presence of various concentrations of PP2. Apoptosis via CD4 was then induced as previously described (II.2.3.9) and measured after 2 h by the PI uptake assay and flow cytometry.

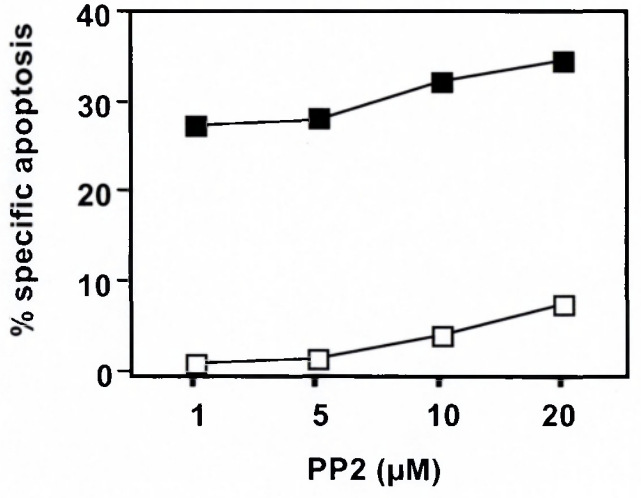

Fig. III.26: PP2 does not inhibit αCD4 induced apoptosis. Specific apoptosis of unstimulated (white squares) or αCD4 stimulated (black squares) HPBALL cells in the presence of various concentrations of PP2 (μM). One representative experiment of three done in triplicates is shown.

PP2 was slightly toxic at high concentrations (7.7 % specific cell death at 20 μM) while no toxicity was observed at concentrations up to 5 μM (figure III.26). PP2 failed to inhibit αCD4 induced apoptosis at all concentrations tested. This further suggested

that the mechanism of αCD4 induced apoptosis does not involve tyrosine phosphorylation of signaling molecules.

6 Role of mitochondria and AIF in αCD4 induced apoptosis

The novel type of caspase independent CD4 and CXCR4 mediated cell death was found to be accompanied by loss of the inner mitochondrial transmembrane potential ($\Delta\Psi m$) (Berndt et al., 1998) which is a result of mitochondrial membrane permeabilisation (MMP). In certain cell types this event is a prerequisite for apoptosis induction. Since mitochondria were also found to be involved in CD4/CXCR4 mediated cell death in PBLs from HIV-1 infected individuals (chapter 2), their role was examined in more depth in the cell line system, focusing on αCD4 induced apoptosis. Moreover, the role of the recently discovered apoptosis inducing factor (AIF) which has been implicated in the induction of caspase independent apoptosis was investigated.

6.1 Mitochondrial transmembrane potential reduction ($\Delta\Psi m$) during αCD4 induced apoptosis is caspase independent

In order to assess whether $\Delta\Psi m$ is a caspase independent event during αCD4 induced apoptosis and thus not due to secondary unspecific effects the pancaspase inhibitor zVAD-fmk was used. This tripeptide broad spectrum caspase inhibitor acts like the closely related inhibitor BD-fmk (see III.2.2.1). HPBALL cells were preincubated for 40 min in the absence or presence of zVAD-fmk. Apoptosis was then induced via CD4 as previously described and $\Delta\Psi m$ was assessed via $DiOC_6(3)$ staining (see II.2.3.7) at the indicated time points (fig.III.27).

As shown in figure III.27 the transmembrane mitochondrial potential was reduced during αCD4 induced apoptosis (A) approximately 30 min after stimulation (B). $\Delta\Psi m$ was not inhibited by zVAD-fmk at any of the time points tested (B).

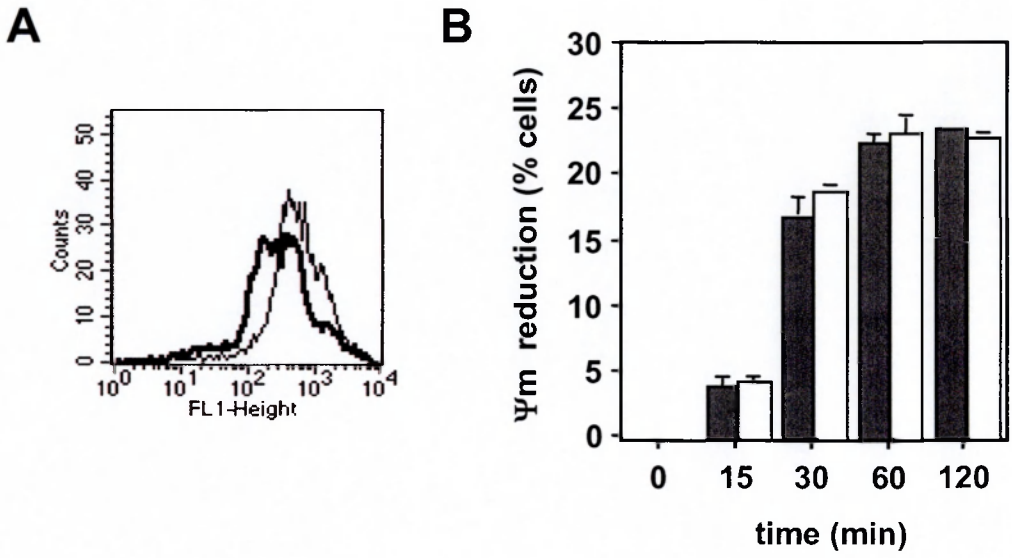

Fig. III.27: ΔΨm during αCD4 induced apoptosis is not inhibited by ZVAD-fmk. (A) Mitochondrial transmembrane potential, evaluated by $DiOC_6(3)$ staining (FL-1), is reduced in αCD4 treated (thick line histogram) compared to untreated (thin line histogram) HPBALL cells. (B) Percentages of HPBALL cells with reduced mitochondrial transmembrane potential after stimulation with αCD4, in the absence (white bars) or presence (grey bars) of 50 μM zVAD-fmk, for the indicated time points. One representative experiment of three done in triplicates is shown.

6.2 Cytochrome C is released during αCD4 induced apoptosis

Since MMP also results in translocation of cytochrome c (Cyt-C) from the mitochondrial intermembrane space to the cytosol (Green and Reed, 1998) Cyt-C localisation was assessed during αCD4 induced apoptosis of HPBALL cells.

CD4 mediated apoptosis was induced as previously described and the samples were then subjected to subcellular fractionation (see II.2.3.8). Cyt-C translocation was then assessed by immunoblotting 1 h after stimulation via αCD4.

As shown in figure III.28A Cyt-C could be seen in the cytosol of untreated cells reflecting the relatively high background apoptosis in these samples (approximately 15 % apoptosis) (lane 1). Upon stimulation via CD4, however, the amount of Cyt-C significantly increased in the cytosolic fraction (compare lanes 1 and 3) reflecting

specific Cyt-C release during αCD4 induced apoptosis. A similar pattern of Cyt-C translocation was seen when the samples were preincubated with the caspase inhibitor zVAD-fmk (fig. 28B). This verified that MMP proceeds in a caspase-independent fashion during CD4 mediated cell death.

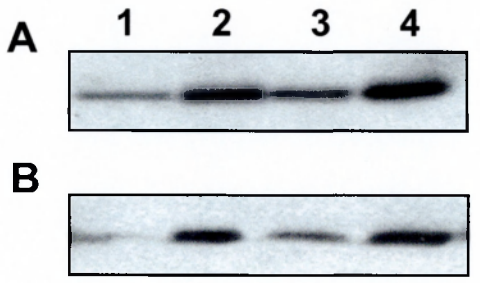

Fig. III.28 : Cytochrome C translocation during αCD4 induced apoptosis. Cytochrome C localisation in cytosolic (lanes 1, 3) or mitochondrial (lanes 2, 4) fractions of αCD4 treated (lanes 3, 4) or untreated (lanes 1, 2) HPBALL cells in the absence (A) or presence (B) of 50 μM ZVAD-fmk.

6.3 AIF tranlocation during αCD4 induced apoptosis

In addition to Cyt-C, the flavoprotein oxidoreductase apoptosis inducing factor (AIF) has been shown to translocate to the cytosol and nucleus thereby stimulating apoptosis via an as yet unknown caspase-independent mechanism (Susin et al., 1999). AIF translocation was evaluated during αCD4 induced apoptosis by intracellular immunofluorescence staining (see II.2.2.3.2). HPBALL cells were incubated in the presence or absence of αCD4 and apoptosis was induced as previously described. After a 2 h incubation the cells were stained with antibodies against AIF and the mitochondrial matrix protein Hsp60 (which does not translocate during apoptosis) as well as with Hoechst 33342 (a DNA intercalating dye). The samples were then analysed by confocal microscopy.

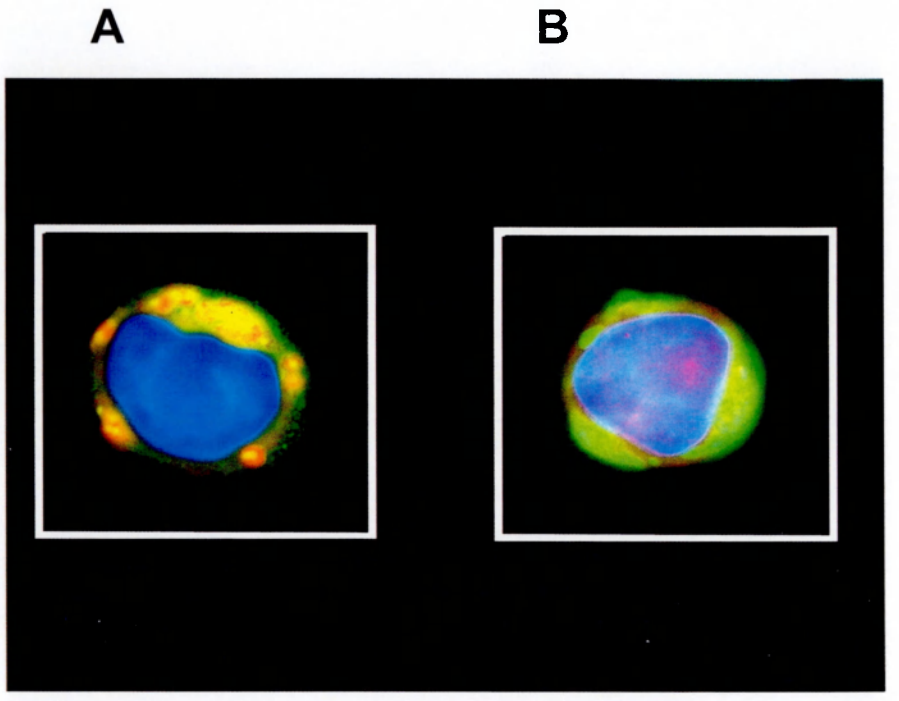

Fig. III.29 : AIF tranlocation during αCD4 induced apoptosis. Untreated (A) or αCD4 treated (B) HPBALL cells were stained with anti-AIF (red fluorescence), anti-Hsp60 (green fluorescence) and Hoechst 33342 (blue fluorescence). AIF colocalises with Hsp60 to the mitochondria (punctate yellow fluorescence) in untreated cells (A), while it translocates to the nucleus and cytosol upon stimulation via CD4 (purple nuclear fluorescence and red fluorescence in the cytosol) (B).

As shown in figure III.29A AIF and Hsp60 were colocalised (yellow punctate fluorescence, blend of green plus red) in control untreated cells. In contrast diffuse red cytoplasmic fluorescence and purple nuclear fluorescence (blend of red plus blue), indicative of the AIF translocation, were observed in anti-CD4 stimulated cells.

6.4 Large scale DNA fragmentation occurs in αCD4 treated HPBALL cells

AIF translocation to the nucleus correlates with the appearance of large scale DNA fragmentation. Since AIF was found to translocate to the nucleus during αCD4 induced apoptosis the formation of large DNA fragments was investigated. CD4 mediated apoptosis was induced as previously described and large scale DNA fragmentation was assessed 3 h or 10 h following stimulation, via pulse field gel electrophoresis (see II.2.3.6).

As shown in figure III.30 large DNA fragmentation (approximately 50 Kb) was readily seen in αCD4 treated cells at both time points tested (lanes B and E). However, when the αCD4 treated cells were preincubated in the presence of the pancaspase inhibitor zVAD-fmk large DNA fragmentation was almost completely inhibited (lanes C and F). Since αCD4 induced apoptosis proceeds in a caspase independent manner this finding suggests that large DNA fragmentation is probably a secondary effect.

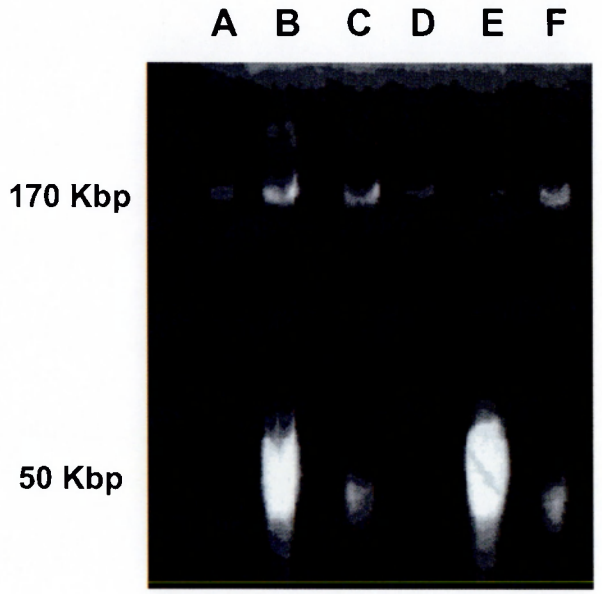

Fig. III.30: Large scale DNA fragmentation occurs in antiCD4 treated HPBALL cells. DNA fragmention pattern of HPBALL cells incubated for 3 h (lanes A, B, C) or 10 h (lanes D, E, F) in the absence (A and D) or presence of either αCD4 (lanes B and E) or αCD4 plus 50 μM ZVAD-fmk (lanes C and F).

IV DISCUSSION

αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals

Patients infected with HIV-1 experience a progressive decline in CD4 T-cell number which leads to immunodeficiency and increased susceptibility to opportunistic infections and malignancies. Although CD4 T-cell production is impaired in HIV-1 infected patients (Hellerstein et al., 1999), the primary basis of T-cell depletion has been shown to be increased apoptosis of CD4 and CD8 T cells (Gougeon et al., 1996; Badley et al., 2000). Since only a minor fraction of apoptotic lymphocytes are physically infected by HIV-1 (Ameisen et al., 1995), cytopathic effects of the virus due to direct infection cannot solely account for the enhanced apoptosis of lymphocytes seen in infected persons. This indicates that other indirect mechanism(s) are involved.

Indeed, several studies have demonstrated that the HIV-1 envelope glycoprotein gp120, either in membrane bound or soluble form, can induce apoptosis of both infected and non-infected bystander T cells (Laurent-Crawford et al., 1993; Finkel et al., 1995; Banda et al., 1992). Moreover crosslinking of the CD4 receptor by monoclonal antibodies, thus mimicking the CD4-gp120 interaction, induces apoptosis in lymphocytes from HIV-1 infected individuals (Oyaizu et al., 1993). Further studies by Westendorp et al. on the mechanism of gp120 induced apoptosis demonstrated that in non-infected lymphocytes this was partly dependent on the CD95 death system. Recent studies, however, showed that stimulation of the CD4 receptor and the CXCR4 chemokine coreceptor by gp120 or monoclonal antibodies could also lead to a novel type of CD95 independent cell death (Berndt et al., 1998).

In this work, the role of CD4 and CXCR4 induced apoptosis as a potential indirect mechanism of T-cell depletion during HIV-1 infection was investigated. Moreover the relative contribution of the previously described cell death pathways

engaged by these receptors during apoptosis induction was assessed in the *in vivo* situation. For the induction of apoptosis and in order to be able to dissect the signals via the two receptors specific antibodies against CD4 and CXCR4 were used recognising the same epitopes as gp120. It was shown that freshly isolated PBLs from a representative cohort of 202 HIV-1 infected individuals exhibited a significantly higher sensitivity to CD4 and CXCR4 mediated apoptosis compared to PBLs from healthy control subjects. Based on these findings the question arises: what does CD4 and CXCR4 mediated cell death represent in the context of HIV-1 infection?

In the context of antigen presentation, antigenic stimulation of lymphocytes in the absence of a costimulatory signal leads to either anergy, in the case of resting cells, or to activation induced cell death, in the case of preactivated cells (Russel et al., 1991; Dhein et al., 1995; Alderson et al., 1995; Miethke et al., 1996). Normally stimulation of the CD4 receptor enhances activation and proliferation that ensues after T-cell receptor (TCR) engagement while stimulation of the CD4 receptor alone (in the absence of TCR engagement) can lead to induction of activation induced apoptosis of preactivated lymphocytes (Algeciras et al., 1998). The same phenomenon has also been described following stimulation of the TCR alone (Dhein et al., 1995); the B-cell receptor (Chan et al., 1990), the MHC-I molecule (Wallen-Ohman et al., 1997; Nilsson et al., 1997), the MHC-II molecule (Truman et al., 1994) as well as the CD2 receptor (Wesselborg et al., 1993; Rouleau et al., 1993).

A general state of immune activation is rapidly observed in the asymptomatic phase of HIV-1 infection in both lymphoid tissue and peripheral blood lymphocytes and persists throughout the course of HIV-1 disease. This is reflected by follicular hyperplasia and expression of activation markers, such as CD45RO in the lymph nodes (Muro-Cacho et al., 1995; Bofill et al., 1995) and in the peripheral blood (Giorgi et al., 1993; Levacher et al., 1992). The degree of lymphocyte apoptosis in lymph nodes from HIV-1 infected patients has been shown to correlate with the state of activation of the lymphoid tissue (Muro-Cacho et al., 1995). Moreover, Gougeon et al. have shown that CD45RO+ activated cells are expanded *in vivo* in HIV-1 infected patients and are more prone to apoptosis compared to the same subset in control subjects.

Therefore, the increased αCD4 induced apoptosis seen in PBLs from HIV-1 infected individuals, in the present experimental system, probably represents a form of activation induced cell death due to the hyperactivation of lymphocytes during the course of the infection. This is further supported by the data obtained with PBLs from healthy individuals where preactivation rendered the cells susceptible to CD4 mediated apoptosis. Subset analysis of these PBLs also showed that CD45RO+, activated, cells were sensitive to CD4 induced apoptosis in contrast to CD45RA+ cells.

In the present system apoptosis was induced via monoclonal antibodies while, in the context of HIV-1 infection circulating immune complexes and replication-incompetent viruses that contain gp120 could induce death in a similar manner (Kameoka et al., 1997; Ellaurie et al., 1990; Aceituno et al., 1997). Moreover, during HIV-1 infection there is an increase of anti-CD4 antibodies (Keay et al.,1995; Muller et al., 1994; Burastero et al., 1996) and more importantly of soluble gp120 (Oh et al.,1992), anti-gp120 antibodies (Ellaurie et al., 1990; Feijoo et al., 1995) and a large number of defective virions (Wie et al., 1995; Ho et al., 1995), all of which can lead to a chronic and significant degree of CD4 activation and subsequent cell death in uninfected CD4+ cells.

In the case of CXCR4 mediated apoptosis a similar scenario could be envisaged. During the course of HIV-1 infection CCR5-using (R5) viruses represent the predominant virus population early after infection while CXCR4-using (X4) viruses and/or viruses with dual tropism emerge later in disease in temporal association with rapid CD4+ T cell decline and progression to AIDS (Connor et al., 1997; Scarlatti et al., 1997; Schuitemaker et al., 1992; Xiao et al., 1998). In fact recent studies have shown that HIV-1 envelope specificity for CXCR4 is linked to aggressive depletion of the CD4+ T-cell population in *ex vivo* human lymphoid histocultures and PBMC suspensions (Penn et al., 1999; Schramm et al., 2000). These findings support the hypothesis that the evolution of viral envelope leading to usage of CXCR4 *in vivo* accelerates loss of CD4+ T cells causing immunodeficiency. It is therefore probable that ligation of CXCR4 in the surface of hyperactivated CD4+ lymphocytes by soluble or

membrane bound gp120 leads to apoptosis in the later stages of HIV-1 infection. As for CD4 mediated apoptosis, this is further supported by the fact that activated CD45RO+ cells from healthy subjects were susceptible to αCXCR4 induced apoptosis in contrast to naive CD45RA+ cells. Moreover CXCR4 was predominantly expressed in CD45RO+ cells which probably accounted for their apoptosis sensitivity.

The findings of this work could have important implications in the development of therapeutic strategies for HIV-1 infection. In view of the critical role of CCR5 and CXCR4 in HIV infection the search and development of blocking agents against these two receptors have been the focus of intensive research. One approach which has been suggested in order to achieve selective blockade of these receptors is the use of monoclonal antibodies. In fact, monoclonal antibodies have been used as effective inhibitors of both chemokine and HIV binding in the case of the CCR3 and CCR5 receptors (Heath et al., 1997; Wu et al., 1997; Grimaldi et al., 1999). However, as the present work shows, antibodies against CXCR4, specific for HIV-1 binding epitopes, could induce cell death and thus may have detrimental effects on the patients.

Therefore, the most promising approach for selective chemokine receptor blockade is represented by small organic molecules. A number of small molecule antagonists have been already identified that are selective for CXCR4 such as AMD3100 (Schols et al., 1997), T22 (Murakami et al., 1997) and ALX40-4C (Doranz et al., 1997). A recent study suggested that ALX40-4C could be safely used in phase I/II clinical trials although more sophisticated measures of viral phenotype were needed to accurately assess its effects (Doranz et al., 2001).

Correlation of CD4/CXCR4 mediated apoptosis with virological features and therapy

Apoptosis induction via CD4 and CXCR4 did not correlate with the plasma viral load, CD4 positive cell counts or stage of the disease of the patients in the overall group of patients tested. Interestingly, however, in patients which did not receive any treatment (therapy naïve), high rates of apoptosis correlated with high viral load and inversely correlated with CD4 counts. This finding further supports the hypothesis that gp120 induces CD4/CXCR4 mediated apoptosis *in vivo* and that this apoptosis plays a role in the pathophysiology of HIV-1 infection, by contributing to the depletion of CD4+ T cells.

Another issue raised, by the findings of this work, is the effect of therapy on CD4 and CXCR4 mediated apoptosis. The introduction of combination therapy has led to an overall drop in mortality and illness due to HIV-1. The HIV protease inhibitors decrease viral replication and hence plasma viral RNA levels resulting in a sustained increase in CD4 positive T cells. Several studies have shown that apoptosis in lymph nodes, rectal mucosa and PBL subsets from patients infected with HIV-1 decreases dramatically in response to protease inhibitor based HIV-1 treatment (Badley et al., 1999; Chavan et al., 1999; Bohler et al., 1999; Johnson et al., 1998; Kotler et al., 1998) . This effect was seen for spontaneous apoptosis, AICD, apoptosis due to mitogenic stimulation and CD95 receptor ligation (Johnson et al., 1998; Aries et al., 1998; Bohler et al., 1999). The data presented in this thesis further support these findings, as HAART initiation led to a significant reduction in CD4/CXCR4 induced apoptosis of PBLs from HIV-1 infected patients, independently of plasma viraemia. It has been previously suggested that protease inhibitors might exert their antiapoptotic function by directly inhibiting caspases (Sloand et al., 2000; Phenix et al., 2000; Weichold et al., 1999). However, recent studies suggest that protease inhibitors may be blocking cell-cycle progression and proteosomal activity in lymphocytes (Chavan et al., 2001; Schmidtke et al., 1999). The predominant apoptotic pathway, engaged during CD4/CXCR4 mediated apoptosis, is caspase independent. This further supports the notion that protease inhibitors may not exert their function by inhibiting caspases.

Multiple cell death pathways are engaged by the CD4 and CXCR4 receptors in PBLs from HIV-1 infected individuals

Since PBLs from HIV-1 infected individuals were sensitive to CD4/CXCR4 mediated cell death it was subsequently investigated whether this phenomenon corresponded to the novel type of apoptotic death previously described to be triggered by these receptors. Initial experiments showed that αCD4 and αCXCR4 induced apoptosis exhibited different kinetics in PBLs from HIV-1 infected individuals than what had been described in cell lines. Thus, maximal apoptosis induction was observed after 18h in PBLs from HIV-1 infected individuals, compared to 2 h in the cell line system. Different kinetics could reflect engagement of different pathways of cell death induction. Alternatively, this could be due to physiological differences between malignant and primary cells such as different stage of activation or sensitization threshold.

In order to gain more insight into the cell death pathways possibly engaged by the CD4 and CXCR4 receptors during apoptosis induction, in the *in vivo* situation, the role of the CD95, TNF and TRAIL systems was assessed. Inhibition of either the CD95 or TNF death systems did not significantly block CD4 and CXCR4 mediated apoptosis in the overall subgroup of patients tested. This is consistent with other studies showing a lack of dependence of HIV-1 induced $CD4^+$ T-cell apoptosis on the CD95 system (Gandhi et al., 1998; Noraz et al., 1997). These findings contradict, however, the studies by Herbein et al. who showed that HIV-1 gp120-CXCR4 interactions triggered T cell death by macrophage production of TNF. In the present system no inhibition of CD4/CXCR4 induced apoptosis was seen by agents blocking the TNF system both in the presence or absence of monocytes.

A partial inhibition of apoptosis was seen by blockage of the TRAIL system. Although the physiological role of the TRAIL receptors remains elusive, previous studies have suggested a role for the TRAIL system in apoptosis in HIV-1 infected patients. T cells from HIV-1 infected patients showed susceptibility to TRAIL mediated apoptosis compared to T cells from healthy control subjects (Jeremias et al., 1998) and

AICD of PBLs from HIV-1 positive individuals could be partially inhibited by TRAIL-specific antagonistic antibodies (Katsikis et al., 1997). The data presented here would further support a role for the TRAIL system in lymphocyte apoptosis during HIV-1 infection, although in the present system this does not represent the principal cell death pathway engaged by the CD4 and CXCR4 receptors. TRAIL induced apoptosis is dependent on caspases. However, in the present system, αCD4/αCXCR4 induced apoptosis could be partially inhibited by inhibitors of the TRAIL system in some patients but not necessarily with caspase inhibitors in the same patients. This would suggest that TRAIL receptor mediated apoptosis can proceed independently of caspases, but it most probably reflects technical limitations of the assays, due to low percentages of apoptosis induction via CD4 and CXCR4 and low percentages of inhibition via the TRAIL system and caspase inhibitors.

Interestingly, the patients that exhibited a partially TRAIL dependent PBL apoptosis had a high viral load . This suggests that different quantitative requirements for gp120 might determine the engagement of different apoptotic pathways.

In order to investigate the role of caspases in CD4/CXCR4 induced apoptosis in HIV-1 infected individuals the broad spectrum, irreversible caspase inhibitor BD-fmk was used. Indeed, in the majority of the patients no inhibition of apoptosis was seen, thus indicating a caspase independent mechanism of apoptosis induction. That was further confirmed by the lack of caspase-8 and PARP cleavage in PBL lysates from patients where apoptosis was not BD-fmk inhibitable. However, in some individuals a partial inhibition via BD-fmk was observed. The inhibition pattern showed great variability within the experimental group. These data suggest that multiple pathways of apoptosis can be engaged by CD4 and CXCR4 in primary cells from HIV-1 infected individuals. As far as CD4 is concerned, this is in agreement with previous findings by Westendorp et al. where CD4 induced apoptosis in primary T cells was partially mediated by a caspase dependent mechanism (Westendorp et al., 1995). Recently, triggering of CXCR4 by either cell surface HIV-1 envelope, soluble primary gp120 or anti-CXCR4 antibodies, in an *ex vivo* infection system, has been demonstrated to induce apoptosis in infected and uninfected primary T cells independently of caspases

(Vlahakis et al., 2001). However, this system might not accurately represent the *in vivo* situation which appears more complex. The data presented in this study suggest that CXCR4 mainly engages a caspase independent pathway of cell death induction but that it also engages a caspase dependent one, albeit to a lesser degree.

Mitochondria have been shown to play a major role in apoptosis triggered by many stimuli. Since mitochondria were shown to be involved in CD4/CXCR4 induced apoptosis in the cell line systems their involvement was also assessed during CD4/CXCR4 induced apoptosis in PBLs from HIV-1 infected patients. Cytochrome c release which results from mitochondrial membrane permeabilisation was seen in CD4/CXCR4 stimulated lymphocytes. However, in this experimental setup it was not possible to define whether cytochrome c release corresponded to the caspase dependent or independent component of CD4/CXCR4 induced apoptosis. The role of mitochondria and cytochrome c release was more extensively examined in the cell line system and will be discussed in further detail in this context.

Another intriguing finding was that no DNA fragmentation, a hallmark of classical apoptosis was seen during CD4/CXCR4 mediated apoptosis. This is in accordance to data obtained in the HPBALL cell line system where no oligonucleosomal DNA fragments were detected during CD4/CXCR4 mediated apoptosis. As previously suggested DNA fragmentation may not be essential for cellular destruction since cytoplasts (cells without nuclei) can undergo apoptotic cell death (Jacobson et al., 1994; Schulze-Osthoff et al., 1994; Nakajima et al., 1995). However, this result should be cautiously interpreted as it could be due to lack of sensitivity of the Nicoletti assay considering the low levels of specific αCD4/αCXCR4 induced apoptosis in PBLs from HIV-1 infected individuals.

Prior to oligonucleosomal DNA fragmentation, large scale DNA fragmentation generating 50 kb fragments also occurs during apoptotic cell death (Wyllie et al., 1980). In fact, in certain systems such as in glucocorticoid induced apoptosis of thymocytes only 50 kb DNA fragments can be detected (Walker et al., 1993). Although large scale DNA fragmentation was not investigated during CD4/CXCR4 mediated apoptosis, in

PBLs from HIV-1 infected individuals, it is probably also not prerequisite for apoptosis induction as shown by experiments in the HPBALL cell line system which will be discussed further on.

Therefore, the findings of this study suggest that the *in vivo* situation is rather complex since multiple cell death pathways seem to be engaged by the CD4 and CXCR4 receptors. Nevertheless, the novel CD95 and caspase independent death pathway seems to be predominantly involved in this phenomenon. The molecular mechanism of this novel pathway was subsequently investigated in cell line systems.

Investigation of the mechanism of CXCR4 mediated apoptosis in a cell line system

In order to identify regions in CXCR4 required for the transmission of the apoptotic signal the ability of chimeras based on CXCR4 and CXCR2 to mediate apoptosis was examined. These receptors share approximately 35% amino acid identity but CXCR2 does not act as a coreceptor for HIV-1. In order to ensure that the apoptotic activity of the chimeric receptors is solely due to the CXCR4 component apoptosis induction was assessed in BJAB cells stably expressing the CXCR2 receptor and stimulated with a monoclonal antibody recognising the amino terminal domain of the receptor. No apoptosis induction was seen following stimulation of the CXCR2 receptor according to the same experimental protocol as for the CXCR4 receptor. However, one cannot exclude that the CXCR2 molecule can potentially mediate apoptosis upon stimulation via antibodies recognising different epitopes.

Nevertheless, based on this approach it was shown that the carboxy terminal domain of CXCR4 was dispensable for apoptosis induction since chimera 4442 and the mutant CXCR4 Δcyto could mediate apoptosis at levels comparable to the wild type receptor (see figure III.17 for schematic representation of chimeric receptors). This indicated that the 18 serine/threonine residues of this domain, which represent potential targets for phosphorylation by G protein-coupled receptor kinases, second messenger-

activated protein kinases and PKC (Freedman and Lefkowitz, 1996) are not involved in this apoptotic pathway. This region of CXCR4 has been implicated in CXCR4 desensitisation and internalisation of the receptor. Deletion of this domain did not affect HIV fusion and entry but prevented the downregulation of the receptor following stimulation via SDF1 (Amara et al., 1997).

The third extracellular loop and seventh transmembrane domain were also dispensable for the apoptotic function of the CXCR4 receptor (chimera 4442). The third extracellular loop has been implicated in the formation and maintenance of a conformation that is permissive for virus interaction although the first and second extracellular loops are the ones critically required for coreceptor activity (Lu et al., 1997). The amino terminal region of CXCR4 (chimera 2444) also did not play a role in apoptosis induction. This was expected as this domain contains the binding site for the natural CXCR4 ligand SDF1 (Crump et al., 1997) which was not found to induce apoptosis in the HPBALL and BJAB cell lines but rather downregulation of the receptor.

Replacement of both the aminoterminal and the region containing the sixth transmembrane domain, third extracellular loop, seventh transmembrane domain and carboxyl terminal (chimera 2442) was also tolerated for apoptosis induction. This indicated that the region from aa 28 till the sixth transmembrane domain was potentially critical for apoptosis induction.

Further replacement of the region of CXCR4 from the amino terminal till the second intracellular loop led to loss of the apoptotic function (chimera 2244) indicating that the first and second intracellular loops are critical for apoptosis induction. The second intracellular loop of CXCR4 contains a DRY amino acid motif which in related receptors plays an important role in G protein coupling (including CCR5) (Lu et al., 1997). Although CXCR4 induced apoptosis was not mediated via the GTPase $G_i\alpha$, other G proteins like $G\alpha_q$ or $G\alpha_{12}$ which have been recently implicated in cell death signaling (Maghazachi, 1997) could play a role in apoptosis induction. Point mutations of this motif and analysis of apoptosis induction will further elucidate its role in cell death signaling.

However the results obtained with the 2244 chimeric receptors should be cautiously interpreted. The 12G5 monoclonal antibody against CXCR4 used to induce apoptosis is conformation dependent and its epitope is contained in extracellular loops one and two. Since extracellular loop one is replaced in 2244, 12G5 fails to bind to the chimeric receptor. Therefore the αCXCR2 antibody 10G2 was used for apoptosis induction via this receptor. The inability of the receptor to transmit the apoptotic signal could reflect stimulation via an inappropriate epitope. Moreover, chimera 2244 was expressed at lower levels on the surface of BJAB cells compared to the wild type receptor which could also explain reduced apoptosis induction.

In general, while much information can be gained by using chimeric receptors the relative importance of specific residues or regions may be masked because of the similarity between the two parental molecules. Moreover, for future studies a slightly different approach could be followed by using receptors where only intracellular parts are exchanged thus retaining the epitope for the apoptotic stimulus.

Investigation of the mechanism of CD4 mediated apoptosis in cell line systems

In order to gain some insight into the mechanism of the novel type of αCD4 induced cell death a structure-function study approach was also followed. BJAB cells expressing truncated forms of CD4 were examined (see figure III.20 for schematic representation of CD4 mutants).

Cells expressing the 431 mutant, lacking two hydrophobic amino acids at the carboxy terminal, were sensitive to αCD4 induced apoptosis. This CD4 mutant has been recently described to confer a gain of function phenotype in antigen dependent T cell activation. Moreover this mutation abolished the binding of the novel CD4 associated protein ACP33 to CD4 (Zeitlmann et al., 2001). Although ACP33 has been suggested to actively transduce an inhibitory signal and therefore act as a negative regulatory factor in CD4 dependent T cell activation, it does not seem to play a role in αCD4 induced

apoptosis. Experiments with the 429 mutant receptor gave similar results as with the 431 receptor indicating that the carboxy terminal proximal region of the receptor is not necessary for apoptosis induction.

The 419 mutant lacks the dicysteine motif (aa 420-422) which harbors the p56lck binding site. Although BJAB cells do not express $p56^{lck}$ this mutant, as well as the p56lck- one, were examined for apoptosis sensitivity as the dicysteine motif could be important for the binding of other B cell expressed src kinases, which could substitute for $p56^{lck}$. Moreover, the association of the adaptor molecule LAT to CD4 has been shown to be promoted by this cysteine motif (Bosselut et al., 1999) which may also be important for B cell expressed adaptor molecules. However, cells expressing the 419 and p56lck- mutants retained sensitivity to αCD4 induced apoptosis thus indicating that this motif is not implicated in the apoptotic signaling.

Expression of the 411 mutant receptor showed that the dileucine motif found in amino acids 413-414 which has been implicated in CD4 signaling was also not involved in the apoptotic pathway (Moutouh et al., 1998). Moreover, the short α helix between aa 409 and 414 which mediates binding of the multimeric adaptor proteins AP-2 and AP-1 was also dispensable for the apoptotic function (Pitcher et al., 1999). These adaptor molecules have been implicated in termination of cell surface receptor signaling and regulation of receptor internalisation (Clements et al., 1999)

Interestingly, apoptosis induction was reduced but not abrogated when the 398 CD4 mutant, lacking the whole cytoplasmic tail, was expressed. This suggested that the cytoplasmic tail of CD4 was dispensable for apoptosis induction although the region from aa 411 to 398, may be implicated in the magnitude of the apoptotic signal.
These results are in agreement with previous studies by Guillerm et al. who found delayed HIV-1 induced apoptosis in cells expressing truncated forms of the CD4 receptor (at aa 401 or 403). This was further supported by the work of Jacotot and coworkers who also reported that A2.01 cells expressing a CD4 mutant receptor truncated at aa 402 underwent apoptosis induced by HIV-1 infection or by coculture with chronically HIV-1 infected H9 cells.

It has also been shown that surface expression of tailless CD4 at high levels is able to circumvent the T cell development requirement for signal transduction by the cytoplasmic domain of CD4 (Killeen and Littman, 1993). However, in the present system, this could not account for the transduction of the apoptotic signal since the 398 receptor was expressed at comparable levels as the wild type CD4.

Further evidence supporting a lack of requirement of the CD4 cytoplasmic tail in αCD4 induced apoptotic signaling was obtained via biochemical analysis of the receptor in the T cell line HPBALL. These data suggested lack of recruitment of signaling molecules to the CD4 receptor complex upon apoptosis induction in metabolically labelled cells. However, this result should be cautiously interpreted as there are some technical pitfalls. Since cellular proteins have variable half lifes one cannot exclude that certain signaling molecules may not be metabolically labelled during the 24 h incubation with [^{35}S] labelled amino acids. Thus, these molecules would not be detected in the experimental set-up used. Moreover, since apoptosis induction requires crosslinking of the αCD4 antibody by a secondary plate bound antibody, it remains unclear to what extend the stimulated CD4 receptor is immunoprecipitated or stays bound to the crosslinking antibody.

Interestingly, however, a quite prominent band of approximately 35 kDa appeared in the unstimulated CD4 receptor complex while it was not detected upon stimulation of the receptor. Whether this represents a negative regulatory factor which is released upon apoptosis induction thus activating downstream signaling molecules needs to be elucidated in future work.

Further biochemical analysis of tyrosine phosphorylation of signaling molecules showed that stimulation of the CD4 receptor via a non-apoptotic stimulus led to phosphorylation of the adaptor molecules LAT and c-Cbl and the protein tyrosine kinase ZAP-70. This is in agreement with other studies where all three molecules have been shown to be phosphorylated upon CD3 or CD4 ligation (van Leeuwen et al., 1999; Murphy et al., 1998). Moreover Cbl has been previously shown to have a negative regulatory role during TCR signaling supporting the notion that CD4 ligation alone leads to negative signaling promoting anergy rather than activation. In contrast, stimulation of the CD4 receptor via an apoptotic stimulus did not lead to particular

phosphorylation events. However, only tyrosine phosphorylation events were examined so it remains to be tested whether there is a role for threonine phosphorylation in the transduction of the apoptotic stimulus. Nevertheless, these results further underscored the lack of requirement of the CD4 cytoplasmic domain in apoptosis signaling.

Since several lines of evidence suggested that the CD4 cytoplasmic tail does not play a role in the transmission of the apoptotic signal it can be envisaged that other domains such as the transmembrane and extracellular may play a role. The immunoglobulin CDR3-like region in domain 1 of CD4 has been suggested to play an important role in CD4 signaling (Briant et al., 1997; Cinek at al., 1995; Friedman et al., 1996). Moreover this region, which is critical for receptor dimerisation, has been implicated in HIV-1 induced apoptosis. A monoclonal antibody which binds to this region was found to inhibit apoptosis mediated by membrane expressed HIV-1 envelope glycoproteins (Guillerm et al., 1998). Whether this domain plays a role in the novel type of αCD4 induced apoptosis needs to be elucidated in future work.

Alternatively, delivery of the apoptotic signals following CD4 stimulation may involve a signal transduction molecule other than CD4. Thus the CD4 molecule may associate via its transmembrane domain another receptor or transmembrane adaptor molecule which then transmits the apoptotic signal. An interesting hypothesis is that the apoptotic signal is trasmitted by the CXCR4 receptor. In such a model CD4 could indirectly trigger HIV-1 induced cell death by allowing optimal interactions between the gp120-gp41 and the CD4-CXCR4 complexes. In the current experimental system apoptosis was triggered via a monoclonal αCD4 antibody. Since there is evidence that association between CXCR4 and CD4 occurs to some extent in the absence of gp120 (Lapham et al., 1996; Ugolini et al., 1997; Kinter et al., 1998; Iyengar et al., 1998), although its presence enhances this association, it is plausible that stimulation of the CD4 receptor suffices to trigger the adjacent CXCR4 receptor. This hypothesis needs to be tested in future experiments.

The studies on CD4 apoptotic signaling were performed in the T cell line HPBALL which expresses CXCR4 but also in the CXCR4 negative B cell line BJAB which was stably transfected with CD4. Since chemokine receptors show great redundancy one cannot exclude that another chemokine receptor expressed in BJAB

cells may compensate for the lack of CXCR4 and can also be found in close proximity to the CD4 receptor.

The role of mitochondria and AIF in the novel type of αCD4 induced apoptosis

Preliminary studies on αCD4 induced apoptosis suggested that the mitochondria may play a role in the execution of the caspase independent apoptotic pathway (Berndt et al., 1998). This was further examined in this study and it was shown that apoptotic cells manifest signs of inner MMP ($\Delta\Psi m$ dissipation) and outer MMP (release of Cyt c) both of which are independent of caspases.

Cytochrome c has been shown to interact with the dATP/ATP binding protein Apaf-1 in a reaction that leads to the autoactivation of pro-caspase-9 which then leads to activation of pro-caspase-3 and initiation of the caspase activation cascade (Liu et al., 1996; Zou et al., 1997). It is therefore intriguing that although cytochrome c is released during αCD4 induced apoptosis caspase-3 and -8 are not activated and the cell death pathway is not inhibitable by the pancaspase inhibitor zVAD-fmk (Berndt et al., 1998). One plausible explanation for this apparent paradox is that the energy status of the apoptotic cell may determine the activation of caspases. It has been previously shown that the complete apoptotic programme involves energy requiring steps, one of wich may be at the level of the formation of the protein complex between Apaf-1, cyt-c and procaspases (Eguchi et al., 1997; Leist et al., 1997). Further pulsed ATP depletion/repletion experiments are needed in order to determine whether a certain energy threshold dictates the activation of caspases in this experimental system.

Alternatively, although inhibitory peptides such as zVAD-fmk are considered specific for the caspase family they may in fact block only some, but not all caspases. Therefore it cannot be excluded that so far unknown caspases or other proteases may be activated following cyt-c release.

The caspase independent effector of apoptosis AIF was also shown to be released from the mitochondria during αCD4 induced apoptosis. Moreover large scale DNA fragmentation was observed which has been previously correlated with AIF translocation to the nucleus (Susin et al., 1999). Paradoxically, formation of large DNA fragments was inhibited by zVAD-fmk. Although in a cell free system and in fibroblastic cell lines AIF causes caspase independent large scale DNA fragmentation and peripheral chromatin condensation (Susin et al., 1999; Daugas et al., 2000), in HIV-1 induced HeLa syncytia the formation of large DNA fragments appears to be fully caspase dependent (Ferri et al., 2000). Therefore, it seems that AIF acts in a cell type specific manner and it remains unclear whether AIF inhibitory factors and/or the abundance of the nuclear AIF target may account for these differences.

Whatever the case may be further experiments are needed using AIF blocking agents in order to prove that AIF is an effector of αCD4 induced apoptosis. Moreover, kinetic analysis of AIF release and nuclear morphological alterations should be performed. In the present experiments AIF translocation was evaluated one hour following CD4 stimulation while large scale DNA fragmentation was evaluated at later time points (3 and 10 h). Since αCD4 induced apoptosis proceeds very rapidly the nuclear fragmentation pattern seen could be due to unspecific activation of caspases secondary to apoptosis.

Kinetic analysis and blocking experiments of cyt-c release should also be performed in the present system, in order to establish an hierarchy of events and define the relative contribution of the mitochondrial factors to apoptosis induction. It has been previously shown in other systems that AIF release precedes that of cyt-c even by several hours (Ferri et al., 2000; Daugas et al., 2000). Since microinjection of recombinant AIF induced MMP and cyt-c release, a caspase independent amplification loop induced by AIF was suggested (Ferri et al., 2000). It is plausible that AIF is rapidly released following CD4 triggering; thus favoring a caspase independent effector pathway; while cytochrome c is released once the cell is in the „commitment" phase. Therefore, even if caspases are activated at that stage, the caspase independent effector pathway may be dominant.

Conclusions

The work described in this thesis aimed at investigating the role of CD4 and CXCR4 induced apoptosis as a potential indirect mechanism of T-cell depletion during HIV-1 infection. The finding that PBLs from HIV-1 infected individuals were sensitive to CD4 and CXCR4 mediated apoptosis suggests a role for this phenomenon in HIV-1 disease pathogenesis. Depletion of CD4+ T cells by such a mechanism is most probably due to the hyperactivation of the immune system seen during the course of the infection. The fact that healthy PBLs could be sensitized , via activation, to undergo CD4 and CXCR4 mediated apoptosis further supports this hypothesis.

Another aim of the work was to investigate the relative contribution of the „classical" or „novel" apoptotic pathways engaged by the CD4 and CXCR4 receptors, upon cell death induction, in the *in vivo* situation. Both pathways were found to be involved, however, the novel caspase independent cell death pathway was the predominant one.

The work also aimed at investigating some of the signaling events involved in the „novel" type of apoptosis triggered by the CD4 and CXCR4 receptors. Studies on CXCR4-CXCR2 receptor chimeras revealed that the first and second intracellular loops of the CXCR4 receptor are probably critical for the propagation of the apoptotic stimulus. Studies on CD4 mutant receptors showed that the cytoplasmic tail of CD4 is dispensable for the induction of cell death. Preliminary biochemical analysis also failed to detect recruitment of signaling molecules to the CD4 receptor complex. These findings thus suggest that a molecule adjacent to the CD4 receptor or the CXCR4 receptor may be responsible for the transmission of the apoptotic signal. Studies on the role of mitochondria during CD4 mediated apoptosis showed release of both cytochrome c and AIF. This suggests a central role for mitochondria in the CD4 induced, caspase independent apoptotic signaling pathway.

Improvement of our understanding of the mechanisms of HIV-1 associated lymphocyte apoptosis, as well as the signaling cascades involved, may lead to

therapeutic strategies aimed at intervening with the CD4+ T cell depletion in HIV-1 infected individuals.

V REFERENCES

Aceituno, E., S. Castanon, C. Jimenez, D. Subira, M. De Gorgolas, M. Fernandez-Guerrero, F. Ortiz, and R. Garcia. 1997. *Circulating immune complexes from HIV-1+ patients induces apoptosis on normal lymphocytes. Immunology* **92**: 317-20.

Adachi, H., G. Preston, B. Harvat, M.I. Dawson, and A.M. Jetten. 1998. *Inhibition of cell proliferation and induction of apoptosis by the retinoid AHPN in human lung carcinoma cells. Am J Respir Cell Mol Biol* **18**: 323-33.

Adams, J.M. and S. Cory. 2001. *Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci* **26**: 61-6.

Alderson, M.R., T.W. Tough, T. Davis-Smith, S. Braddy, B. Falk, K.A. Schooley, R.G. Goodwin, C.A. Smith, F. Ramsdell, and D.H. Lynch. 1995. *Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med* **181**: 71-7.

Algeciras, A., D.H. Dockrell, D.H. Lynch, and C.V. Paya. 1998. *CD4 regulates susceptibility to Fas ligand- and tumor necrosis factor- mediated apoptosis. J Exp Med* **187**: 711-20.

Alnemri, E.S., D.J. Livingston, D.W. Nicholson, G. Salvesen, N.A. Thornberry, W.W. Wong, and J. Yuan. 1996. *Human ICE/CED-3 protease nomenclature. Cell* **87**: 171.

Amara, A., S.L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggiolini, J.L. Virelizier, and F. Arenzana-Seisdedos. 1997. *HIV coreceptor downregulation as antiviral principle: SDF-1alpha- dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J Exp Med* **186**: 139-46.

Amarante-Mendes, G.P., D.M. Finucane, S.J. Martin, T.G. Cotter, G.S. Salvesen, and D.R. Green. 1998. *Anti-apoptotic oncogenes prevent caspase-dependent and independent commitment for cell death. Cell Death Differ* **5**: 298-306.

Ameisen, J.C., J. Estaquier, T. Idziorek, and F. De Bels. 1995. *Programmed cell death and AIDS pathogenesis: significance and potential mechanisms. Curr Top Microbiol Immunol* **200**: 195-211.

Aries, S. P.,Weyrich, K., Schaaf, B., Hansen, F., Dennin, R.H., and Dalhoff, K. 1998. *Early T-cell apoptosis and Fas expression during antiretroviral therapy in individuals infected with human immunodeficiency virus-1. Scand J Immunol* **48**: 86-91.

Badley, A.D., D.H. Dockrell, A. Algeciras, S. Ziesmer, A. Landay, M.M. Lederman, E. Connick, H. Kessler, D. Kuritzkes, D.H. Lynch, P. Roche, H. Yagita, and C.V. Paya. 1998. *In vivo analysis of Fas/FasL interactions in HIV-infected patients. J Clin Invest* **102**: 79-87.

Badley, A.D., K. Parato, D.W. Cameron, S. Kravcik, B.N. Phenix, D. Ashby, A. Kumar, D.H. Lynch, J. Tschopp, and J.B. Angel. 1999. *Dynamic correlation of apoptosis and immune activation during treatment of HIV infection. Cell Death Differ* **6**: 420-32.

Badley, A.D., A.A. Pilon, A. Landay, and D.H. Lynch. 2000. *Mechanisms of HIV-associated lymphocyte apoptosis. Blood* **96**: 2951-64.

Baggiolini, M., B. Dewald, and B. Moser. 1997. *Human chemokines: an update. Annu Rev Immunol* **15**: 675-705.

Banda, N.K., J. Bernier, D.K. Kurahara, R. Kurrle, N. Haigwood, R.P. Sekaly, and T.H. Finkel. 1992. *Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J Exp Med* **176**: 1099-106.

Baumler, C.B., T. Bohler, I. Herr, A. Benner, P.H. Krammer, and K.M. Debatin. 1996. *Activation of the CD95 (APO-1/Fas) system in T cells from human immunodeficiency virus type-1-infected children. Blood* **88**: 1741-6.

Beidler, D.R., M. Tewari, P.D. Friesen, G. Poirier, and V.M. Dixit. 1995. *The baculovirus p35 protein inhibits Fas- and tumor necrosis factor- induced apoptosis. J Biol Chem* **270**: 16526-8.

Benson, R.S., C. Dive, and A.J. Watson. 1998. *Comparative effects of Bcl-2 over-expression and ZVAD.FMK treatment on dexamethasone and VP16-induced apoptosis in CEM cells. Cell Death Differ* **5**: 432-9.

Berger, E.A. 1997. *HIV entry and tropism: the chemokine receptor connection. Aids* **11**: S3-16.

Berndt, C., B. Mopps, S. Angermuller, P. Gierschik, and P.H. Krammer. 1998. *CXCR4 and CD4 mediate a rapid CD95-independent cell death in CD4(+) T cells. Proc Natl Acad Sci U S A* **95**: 12556-61.

Berridge, M.J., M.D. Bootman, and P. Lipp. 1998. *Calcium--a life and death signal. Nature* **395**: 645-8.

Biard-Piechaczyk, M., V. Robert-Hebmann, V. Richard, J. Roland, R.A. Hipskind, and C. Devaux. 2000. *Caspase-dependent apoptosis of cells expressing the chemokine receptor CXCR4 is induced by cell membrane-associated human immunodeficiency virus type 1 envelope glycoprotein (gp120). Virology* **268**: 329-44.

Biti, R., R. Ffrench, J. Young, B. Bennetts, G. Stewart, and T. Liang. 1997. *HIV-1 infection in an individual homozygous for the CCR5 deletion allele [letter; comment]. Nat Med* **3**: 252-3.

Blanco, J., E. Jacotot, C. Cabrera, A. Cardona, B. Clotet, E. De Clercq, and J.A. Este. 1999. *The implication of the chemokine receptor CXCR4 in HIV-1 envelope protein-induced apoptosis is independent of the G protein-mediated signalling. Aids* **13**: 909-17.

Bofill, M., W. Gombert, N.J. Borthwick, A.N. Akbar, J.E. McLaughlin, C.A. Lee, M.A. Johnson, A.J. Pinching, and G. Janossy. 1995. *Presence of CD3+CD8+Bcl-2(low) lymphocytes undergoing apoptosis and activated macrophages in lymph nodes of HIV-1+ patients. Am J Pathol* **146**: 1542-55.

Bohler, T., J. Walcher, G. Holzl-Wenig, M. Geiss, B. Buchholz, R. Linde and K.M. Debatin. 1999. *Early effects of antiretroviral combination therapy on activation, apoptosis and regeneration of T cells in HIV-1 infected children and adolescents. AIDS* **13:** 779-89

Boldin, M.P., E.E. Varfolomeev, Z. Pancer, I.L. Mett, J.H. Camonis, and D. Wallach. 1995. *A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J Biol Chem* **270**: 7795-8.

Borner, C. and L. Monney. 1999. *Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ* **6**: 497-507.

Borrow, P., H. Lewicki, B.H. Hahn, G.M. Shaw, and M.B. Oldstone. 1994. *Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol* **68**: 6103-10.

Bosselut, R., W. Zhang, J.M. Ashe, J.L. Kopacz, L.E. Samelson, and A. Singer. 1999. *Association of the adaptor molecule LAT with CD4 and CD8 coreceptors identifies a new coreceptor function in T cell receptor signal transduction. J Exp Med* **190**: 1517-26.

Briant, L., N. Signoret, M. Gaubin, V. Robert-Hebmann, X. Zhang, R. Murali, M.I. Greene, D. Piatier-Tonneau, and C. Devaux. 1997. *Transduction of activation signal that follows HIV-1 binding to CD4 and CD4 dimerization involves the immunoglobulin CDR3-like region in domain 1 of CD4. J Biol Chem* **272**: 19441-50.

Brojatsch, J., J. Naughton, M.M. Rolls, K. Zingler, and J.A. Young. 1996. *CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. Cell* **87**: 845-55.

Brown, T.L., S. Patil, R.K. Basnett, and P.H. Howe. 1998. *Caspase inhibitor BD-fmk distinguishes transforming growth factor beta- induced apoptosis from growth inhibition. Cell Growth Differ* **9**: 869-75.

Brunet, C.L., R.H. Gunby, R.S. Benson, J.A. Hickman, A.J. Watson, and G. Brady. 1998. *Commitment to cell death measured by loss of clonogenicity is separable from the appearance of apoptotic markers. Cell Death Differ* **5**: 107-15.

Buchbinder, S.P., M.H. Katz, N.A. Hessol, P.M. O'Malley, and S.D. Holmberg. 1994. *Long-term HIV-1 infection without immunologic progression. Aids* **8**: 1123-8.

Buendia, B., A. Santa-Maria, and J.C. Courvalin. 1999. *Caspase-dependent proteolysis of integral and peripheral proteins of nuclear membranes and nuclear pore complex proteins during apoptosis. J Cell Sci* **112**: 1743-53.

Burastero, S.E., D. Gaffi, L. Lopalco, G. Tambussi, B. Borgonovo, C. De Santis, C. Abecasis, P. Robbioni, A. Gasparri, A. Lazzarin, F. Celada, A.G. Siccardi, and A. Beretta. 1996. *Autoantibodies to CD4 in HIV type 1-exposed seronegative individuals. AIDS Res Hum Retroviruses* **12**: 273-80.

Carrera, A.C., F. Sanchez-Madrid, M. Lopez-Botet, C. Bernabeu, and M.O. De Landazuri. 1987. *Involvement of the CD4 molecule in a post-activation event on T cell proliferation. Eur J Immunol* **17**: 179-86.

Casciola-Rosen, L.A., G.J. Anhalt, and A. Rosen. 1995. *DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. J Exp Med* **182**: 1625-34.

Casciola-Rosen, L.A., D.K. Miller, G.J. Anhalt, and A. Rosen. 1994. *Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. J Biol Chem* **269**: 30757-60.

Castro, K.G. 1993. *Distribution of acquired immunodeficiency syndrome and other sexually transmitted diseases in racial and ethnic populations, United States. Influences of life-style and socioeconomic status. Ann Epidemiol* **3**: 181-4.

Chan, C.S., F. Soehnlen, and G.P. Schechter. 1990. *Differential response of malignant human B-cells to anti-IgM immunoglobulin (anti-mu) and B-cell growth factor: unique direct cytotoxicity of anti-mu on prolymphocytic leukemia cells. Blood* **76**: 1601-6.

Chang, T.W. and H.N. Eisen. 1980. *Effects of N alpha-tosyl-L-lysyl-chloromethylketone on the activity of cytotoxic T lymphocytes. J Immunol* **124**: 1028-33.

Chavan , S., S.L. Tamma, M. Gersten, and S.G. Pahwa. 1999. *Reduction in T cell apoptosis in patients with HIV disease following antiretroviral therapy. Clin Immunol* **93:** 24-33

Chavan , S., S. Kodoth, R. Pahwa and S.G. Pahwa. 2001. *The HIV protease inhibitor Indinavir inhibits cell-cycle progression in vitro in lymphocytes of HIV-infected and uninfected individuals. Blood* **15:** 383-9

Chicheportiche, Y., P.R. Bourdon, H. Xu, Y.M. Hsu, H. Scott, C. Hession, I. Garcia, and J.L. Browning. 1997. *TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. J Biol Chem* **272**: 32401-10.

Chinnaiyan, A.M., K. O'Rourke, M. Tewari, and V.M. Dixit. 1995. *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell* **81**: 505-12.

Chinnaiyan, A.M., K. O'Rourke, G.L. Yu, R.H. Lyons, M. Garg, D.R. Duan, L. Xing, R. Gentz, J. Ni, and V.M. Dixit. 1996. *Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. Science* **274**: 990-2.

Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P.D. Ponath, L. Wu, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. *The*

beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85**: 1135-48.

Chun, T.W., L. Carruth, D. Finzi, X. Shen, J.A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T.C. Quinn, Y.H. Kuo, R. Brookmeyer, M.A. Zeiger, P. Barditch-Crovo, and R.F. Siliciano. 1997. *Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature* **387**: 183-8.

Cinek, T., I. Hilgert, and V. Horejsi. 1995. *An alternative way of CD4 and CD8 association with protein kinases of the Src family. Immunogenetics* **41**: 110-6.

Clements, J.L., N.J. Boerth, J.R. Lee, and G.A. Koretzky. 1999. *Integration of T cell receptor-dependent signaling pathways by adapter proteins. Annu Rev Immunol* **17**: 89-108.

Cocchi, F., A.L. DeVico, A. Garzino-Demo, A. Cara, R.C. Gallo, and P. Lusso. 1996. *The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. Nat Med* **2**: 1244-7.

Connor, R.I., H. Mohri, Y. Cao, and D.D. Ho. 1993. *Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in human immunodeficiency virus type 1-infected individuals. J Virol* **67**: 1772-7.

Connor, R.I., W.A. Paxton, K.E. Sheridan, and R.A. Koup. 1996. *Macrophages and CD4+ T lymphocytes from two multiply exposed, uninfected individuals resist infection with primary non-syncytium- inducing isolates of human immunodeficiency virus type 1. J Virol* **70**: 8758-64.

Connor, R.I., K.E. Sheridan, D. Ceradini, S. Choe, and N.R. Landau. 1997. *Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J Exp Med* **185**: 621-8.

Corbeil, J. and D.D. Richman. 1995. *Productive infection and subsequent interaction of CD4-gp120 at the cellular membrane is required for HIV-induced apoptosis of CD4+ T cells. J Gen Virol* **76**: 681-90.

Crump, M.P., J.H. Gong, P. Loetscher, K. Rajarathnam, A. Amara, F. Arenzana-Seisdedos, J.L. Virelizier, M. Baggiolini, B.D. Sykes, and I. Clark-Lewis. 1997. *Solution structure and basis for functional activity of stromal cell- derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. Embo J* **16**: 6996-7007.

Daar, E.S., T. Moudgil, R.D. Meyer, and D.D. Ho. 1991. *Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection. N Engl J Med* **324**: 961-4.

Daugas, E., D. Nochy, L. Ravagnan, M. Loeffler, S.A. Susin, N. Zamzami, and G. Kroemer. 2000a. *Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. FEBS Lett* **476**: 118-23.

Daugas, E., S.A. Susin, N. Zamzami, K.F. Ferri, T. Irinopoulou, N. Larochette, M.C. Prevost, B. Leber, D. Andrews, J. Penninger, and G. Kroemer. 2000b. *Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. Faseb J* **14**: 729-39.

Davis, C.B., I. Dikic, D. Unutmaz, C.M. Hill, J. Arthos, M.A. Siani, D.A. Thompson, J. Schlessinger, and D.R. Littman. 1997. *Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. J Exp Med* **186**: 1793-8.

De Maria, R., L. Lenti, F. Malisan, F. d'Agostino, B. Tomassini, A. Zeuner, M.R. Rippo, and R. Testi. 1997. *Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science* **277**: 1652-5.

Dean, M., M. Carrington, C. Winkler, G.A. Huttley, M.W. Smith, R. Allikmets, J.J. Goedert, S.P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels, and S.J. O'Brien. 1996. *Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science* **273**: 1856-62.

Deas, O., C. Dumont, M. MacFarlane, M. Rouleau, C. Hebib, F. Harper, F. Hirsch, B. Charpentier, G.M. Cohen, and A. Senik. 1998. *Caspase-independent cell death induced by anti-CD2 or staurosporine in activated human peripheral T lymphocytes. J Immunol* **161**: 3375-83.

Debatin, K.M., A. Fahrig-Faissner, S. Enenkel-Stoodt, W. Kreuz, A. Benner, and P.H. Krammer. 1994. *High expression of APO-1 (CD95) on T lymphocytes from human immunodeficiency virus-1-infected children [letter]. Blood* **83**: 3101-3.

Deiss, L.P., H. Galinka, H. Berissi, O. Cohen, and A. Kimchi. 1996. *Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. Embo J* **15**: 3861-70.

Dhein, J., H. Walczak, C. Baumler, K.M. Debatin, and P.H. Krammer. 1995. *Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature* **373**: 438-41.

Doranz, B.J., L.G. Filion, F. Diaz-Mitoma, D.S. Sitar, J. Sahai, F. Baribaud, M.J. Orsini, J.L. Benovic, W. Cameron, and R.W. Doms. 2001. *Safe use of the CXCR4 inhibitor ALX40-4C in humans. AIDS Res Hum Retroviruses* **17**: 475-86.

Doranz, B.J., K. Grovit-Ferbas, M.P. Sharron, S.H. Mao, M.B. Goetz, E.S. Daar, R.W. Doms, and W.A. O'Brien. 1997. *A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. J Exp Med* **186**: 1395-400.

Doranz, B.J., J. Rucker, Y. Yi, R.J. Smyth, M. Samson, S.C. Peiper, M. Parmentier, R.G. Collman, and R.W. Doms. 1996. *A dual-tropic primary HIV-1 isolate that uses fusin and the beta- chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell* **85**: 1149-58.

Dümbgen, L. 1991. *The asymptomatic behaviour of some nonparametric change-point estimators. The Annals of Statistics* **19**: 1471-1495

Eguchi, Y., S. Shimizu, and Y. Tsujimoto. 1997. *Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res* **57**: 1835-40.

Ellaurie, M., T.A. Calvelli, and A. Rubinstein. 1990. *Human immunodeficiency virus (HIV) circulating immune complexes in infected children. AIDS Res Hum Retroviruses* **6**: 1437-41.

Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. *A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature* **391**: 43-50.

Farzan, M., H. Choe, K.A. Martin, Y. Sun, M. Sidelko, C.R. Mackay, N.P. Gerard, J. Sodroski, and C. Gerard. 1997. *HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5. J Biol Chem* **272**: 6854-7.

Feijoo, E., D. Subira, M. Fernandez-Guerrero, R. Garcia, and F. Ortiz. 1995. *Anti-CD4 activity in circulating immune complexes in HIV-infected patients. Int Arch Allergy Immunol* **106**: 366-71.

Feng, Y., C.C. Broder, P.E. Kennedy, and E.A. Berger. 1996. *HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor [see comments]. Science* **272**: 872-7.

Ferri, K.F., E. Jacotot, J. Blanco, J.A. Este, N. Zamzami, S.A. Susin, Z. Xie, G. Brothers, J.C. Reed, J.M. Penninger, and G. Kroemer. 2000. *Apoptosis control in syncytia induced by the HIV type 1-envelope glycoprotein complex. Role Of mitochondria and caspases [In Process Citation]. J Exp Med* **192**: 1081-92.

Finkel, T.H., G. Tudor-Williams, N.K. Banda, M.F. Cotton, T. Curiel, C. Monks, T.W. Baba, R.M. Ruprecht, and A. Kupfer. 1995. *Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes [see comments]. Nat Med* **1**: 129-34.

Fouchier, R.A., M. Groenink, N.A. Kootstra, M. Tersmette, H.G. Huisman, F. Miedema, and H. Schuitemaker. 1992. *Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol* **66**: 3183-7.

Fox, C.H. 1987. *AIDS caused by a slow virus. Nature* **326**: 636.

Freedman, N.J. and R.J. Lefkowitz. 1996. *Desensitization of G protein-coupled receptors. Recent Prog Horm Res* **51**: 319-51.

Friedman, T.M., A.P. Reddy, R. Wassell, B.A. Jameson, and R. Korngold. 1996. *Identification of a human CD4-CDR3-like surface involved in CD4+ T cell function. J Biol Chem* **271**: 22635-40.

Gagliardini, V., P.A. Fernandez, R.K. Lee, H.C. Drexler, R.J. Rotello, M.C. Fishman, and J. Yuan. 1994. *Prevention of vertebrate neuronal death by the crmA gene. Science* **263**: 826-8.

Gandhi, R.T., B.K. Chen, S.E. Straus, J.K. Dale, M.J. Lenardo, and D. Baltimore. 1998. *HIV-1 directly kills CD4+ T cells by a Fas-independent mechanism. J Exp Med* **187**: 1113-22.

Ganju, R.K., P. Dutt, L. Wu, W. Newman, H. Avraham, S. Avraham, and J.E. Groopman. 1998. *Beta-chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. Blood* **91**: 791-7.

Garcia-Calvo, M., E.P. Peterson, B. Leiting, R. Ruel, D.W. Nicholson, and N.A. Thornberry. 1998. *Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem* **273**: 32608-13.

Giorgi, J.V., Z. Liu, L.E. Hultin, W.G. Cumberland, K. Hennessey, and R. Detels. 1993. *Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. J Acquir Immune Defic Syndr* **6**: 904-12.

Goudsmit, J. 1995. *The role of viral diversity in HIV pathogenesis. J Acquir Immune Defic Syndr Hum Retrovirol* **10**: S15-9.

Gougeon, M.L., S. Garcia, J. Heeney, R. Tschopp, H. Lecoeur, D. Guetard, V. Rame, C. Dauguet, and L. Montagnier. 1993. *Programmed cell death in AIDS-related HIV and SIV infections. AIDS Res Hum Retroviruses* **9**: 553-63.

Gougeon, M.L., H. Lecoeur, A. Dulioust, M.G. Enouf, M. Crouvoiser, C. Goujard, T. Debord, and L. Montagnier. 1996. *Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. J Immunol* **156**: 3509-20.

Gougeon, M.L. and L. Montagnier. 1993. *Apoptosis in AIDS [published erratum appears in Science 1993 Jun 18;260(5115):1709] [see comments]. Science* **260**: 1269-70.

Green, D.R. and J.C. Reed. 1998. *Mitochondria and apoptosis. Science* **281**: 1309-12.

Grimaldi, J.C., N.X. Yu, G. Grunig, B.W. Seymour, F. Cottrez, D.S. Robinson, N. Hosken, W.G. Ferlin, X. Wu, H. Soto, A. O'Garra, M.C. Howard, and R.L. Coffman. 1999. *Depletion of eosinophils in mice through the use of antibodies specific for C-C chemokine receptor 3 (CCR3). J Leukoc Biol* **65**: 846-53.

Gross, A., J. Jockel, M.C. Wei, and S.J. Korsmeyer. 1998. *Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. Embo J* **17**: 3878-85.

Gross, A., J.M. McDonnell, and S.J. Korsmeyer. 1999. *BCL-2 family members and the mitochondria in apoptosis. Genes Dev* **13**: 1899-911.

Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J.C. Ameisen. 1992. *Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. J Exp Med* **175**: 331-40.

Grube, K. and A. Burkle. 1992. *Poly(ADP-ribose) polymerase activity in mononuclear leukocytes of 13 mammalian species correlates with species-specific life span. Proc Natl Acad Sci U S A* **89**: 11759-63.

Gu, Y., C. Sarnecki, R.A. Aldape, D.J. Livingston, and M.S. Su. 1995. *Cleavage of poly(ADP-ribose) polymerase by interleukin-1 beta converting enzyme and its homologs TX and Nedd-2. J Biol Chem* **270**: 18715-8.

Guillerm, C., N. Coudronniere, V. Robert-Hebmann, and C. Devaux. 1998. *Delayed human immunodeficiency virus type 1-induced apoptosis in cells expressing truncated forms of CD4. J Virol* **72**: 1754-61.

Ha, H.C., P.M. Woster, and R.A. Casero, Jr. 1998. *Unsymmetrically substituted polyamine analogue induces caspase- independent programmed cell death in Bcl-2-overexpressing cells. Cancer Res* **58**: 2711-4.

Han, X., K. Becker, H.J. Degen, H. Jablonowski, and G. Strohmeyer. 1996. *Synergistic stimulatory effects of tumour necrosis factor alpha and interferon gamma on replication of human immunodeficiency virus type 1 and on apoptosis of HIV-1-infected host cells. Eur J Clin Invest* **26**: 286-92.

Heath, H., S. Qin, P. Rao, L. Wu, G. LaRosa, N. Kassam, P.D. Ponath, and C.R. Mackay. 1997. *Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest* **99**: 178-84.

Heeney, J.L. 1995. *AIDS: a disease of impaired Th-cell renewal? Immunol Today* **16**: 515-20.

Hellerstein, M., M.B. Hanley, D. Cesar, S. Siler, C. Papageorgopoulos, E. Wieder, D. Schmidt, R. Hoh, R. Neese, D. Macallan, S. Deeks, and J.M. McCune. 1999. *Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. Nat Med* **5**: 83-9.

Herbein, G., U. Mahlknecht, F. Batliwalla, P. Gregersen, T. Pappas, J. Butler, W.A. O'Brien, and E. Verdin. 1998. *Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4 [see comments]. Nature* **395**: 189-94.

Hesselgesser, J., D. Taub, P. Baskar, M. Greenberg, J. Hoxie, D.L. Kolson, and R. Horuk. 1998. *Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. Curr Biol* **8**: 595-8.

Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard, and M. Markowitz. 1995. *Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature* **373**: 123-6.

Holler, N., R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J.L. Bodmer, P. Schneider, B. Seed, and J. Tschopp. 2000. *Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol* **1**: 489-95.

Hughes, F.M., Jr., R.B. Evans-Storms, and J.A. Cidlowski. 1998. *Evidence that non-caspase proteases are required for chromatin degradation during apoptosis. Cell Death Differ* **5**: 1017-27.

Iyengar, S., J.E. Hildreth, and D.H. Schwartz. 1998. *Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. J Virol* **72**: 5251-5.

Jacobson, M.D., J.F. Burne, and M.C. Raff. 1994. *Programmed cell death and Bcl-2 protection in the absence of a nucleus. Embo J* **13**: 1899-910.

Jacotot, E., B. Krust, C. Callebaut, A. G. Laurent-Crawford, J. Blanco, and A. G. Hovanessian. 1997. *HIV-1 envelope glycoproteins-mediated apoptosis is regulated by CD4 dependent and independent mechanisms. Apoptosis* **2**: 47-60

Jacotot, E., L. Ravagnan, M. Loeffler, K.F. Ferri, H.L. Vieira, N. Zamzami, P. Costantini, S. Druillennec, J. Hoebeke, J.P. Briand, T. Irinopoulou, E. Daugas, S.A. Susin, D. Cointe, Z.H. Xie, J.C. Reed, B.P. Roques, and G. Kroemer. 2000. *The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. J Exp Med* **191**: 33-46.

Jeremias, I., I. Herr, T. Boehler, and K.M. Debatin. 1998. *TRAIL/Apo-2-ligand-induced apoptosis in human T cells. Eur J Immunol* **28**: 143-52.

Johnson, N., and Parkin, J. M. 1998. *Anti-retroviral therapy reverses HIV-associated abnormalities in lymphocyte apoptosis. Clin Exp Immunol* **113**: 229-34.

Kameoka, M., T. Kimura, Y.H. Zheng, S. Suzuki, K. Fujinaga, R.B. Luftig, and K. Ikuta. 1997. *Protease-defective, gp120-containing human immunodeficiency virus type 1 particles induce apoptosis more efficiently than does wild-type virus or recombinant gp120 protein in healthy donor-derived peripheral blood T cells. J Clin Microbiol* **35**: 41-7.

Kamita, S.G., K. Majima, and S. Maeda. 1993. *Identification and characterization of the p35 gene of Bombyx mori nuclear polyhedrosis virus that prevents virus-induced apoptosis. J Virol* **67**: 455-63.

Katsikis, P.D., M.E. Garcia-Ojeda, J.F. Torres-Roca, I.M. Tijoe, C.A. Smith, and L.A. Herzenberg. 1997. *Interleukin-1 beta converting enzyme-like protease involvement in Fas- induced and activation-induced peripheral blood T cell apoptosis in HIV infection. TNF-related apoptosis-inducing ligand can mediate activation- induced T cell death in HIV infection. J Exp Med* **186**: 1365-72.

Keay, S., W. Wecksler, S.S. Wasserman, J. Margolick, and H. Farzadegan. 1995. *Association between anti-CD4 antibodies and a decline in CD4+ lymphocytes in human immunodeficiency virus type 1 seroconverters. J Infect Dis* **171**: 312-9.

Kerr, J.F., A.H. Wyllie, and A.R. Currie. 1972. *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer* **26**: 239-57.

Killeen, N. and D.R. Littman. 1993. *Helper T-cell development in the absence of CD4-p56lck association. Nature* **364**: 729-32.

Kinter, A., A. Catanzaro, J. Monaco, M. Ruiz, J. Justement, S. Moir, J. Arthos, A. Oliva, L. Ehler, S. Mizell, R. Jackson, M. Ostrowski, J. Hoxie, R. Offord, and A.S. Fauci. 1998. *CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: role of signal transduction. Proc Natl Acad Sci U S A* **95**: 11880-5.

Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Krammer, and M.E. Peter. 1995. *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. Embo J* **14**: 5579-88.

Kitson, J., T. Raven, Y.P. Jiang, D.V. Goeddel, K.M. Giles, K.T. Pun, C.J. Grinham, R. Brown, and S.N. Farrow. 1996. *A death-domain-containing receptor that mediates apoptosis. Nature* **384**: 372-5.

Kolesnitchenko, V., L. King, A. Riva, Y. Tani, S.J. Korsmeyer, and D.I. Cohen. 1997. *A major human immunodeficiency virus type 1-initiated killing pathway distinct from apoptosis. J Virol* **71**: 9753-63.

Komiyama, T., C.A. Ray, D.J. Pickup, A.D. Howard, N.A. Thornberry, E.P. Peterson, and G. Salvesen. 1994. *Inhibition of interleukin-1 beta converting enzyme by the cowpox virus serpin CrmA. An example of cross-class inhibition. J Biol Chem* **269**: 19331-7.

Kothakota, S., T. Azuma, C. Reinhard, A. Klippel, J. Tang, K. Chu, T.J. McGarry, M.W. Kirschner, K. Koths, D.J. Kwiatkowski, and L.T. Williams. 1997. *Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science* **278**: 294-8.

Kotler, D.P., Shimada, T., Snow, G., Winson, G., Chen, W., Zhao, M., Inada, Y., Clayton, F. 1998. *Effect of combination antiretroviral therapy upon rectal mucosal HIV RNA burden and mononuclear cell apoptosis. Aids* **12**: 597-604.

Krammer, P.H. 2000. *CD95's deadly mission in the immune system. Nature* **407**: 789-95.

Kroemer, G. 1995. *The pharmacology of T cell apoptosis. Adv Immunol* **58**: 211-96.

Kroemer, G., B. Dallaporta, and M. Resche-Rigon. 1998. *The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol* **60**: 619-42.

Kroemer, G. and J.C. Reed. 2000. *Mitochondrial control of cell death. Nat Med* **6**: 513-9.

Laemmli, U.K. 1970. *Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature* **227**: 680-5.

Lapham, C.K., J. Ouyang, B. Chandrasekhar, N.Y. Nguyen, D.S. Dimitrov, and H. Golding. 1996. *Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. Science* **274**: 602-5.

Laurent-Crawford, A.G., B. Krust, Y. Riviere, C. Desgranges, S. Muller, M.P. Kieny, C. Dauguet, and A.G. Hovanessian. 1993. *Membrane expression of HIV envelope glycoproteins triggers apoptosis in CD4 cells. AIDS Res Hum Retroviruses* **9**: 761-73.

Lavoie, J.N., M. Nguyen, R.C. Marcellus, P.E. Branton, and G.C. Shore. 1998. *E4orf4, a novel adenovirus death factor that induces p53-independent apoptosis by a pathway that is not inhibited by zVAD-fmk. J Cell Biol* **140**: 637-45.

Lazebnik, Y.A., S.H. Kaufmann, S. Desnoyers, G.G. Poirier, and W.C. Earnshaw. 1994. *Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature* **371**: 346-7.

Lehmann, E.L. 1975. *Nonparametrics: Statistical Methods Based on Ranks.* Oakland, Calif.: Holden-Day

Leist, M., B. Single, A.F. Castoldi, S. Kuhnle, and P. Nicotera. 1997. *Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J Exp Med* **185**: 1481-6.

Levacher, M., F. Hulstaert, S. Tallet, S. Ullery, J.J. Pocidalo, and B.A. Bach. 1992. *The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. Clin Exp Immunol* **90**: 376-82.

Li, H., H. Zhu, C.J. Xu, and J. Yuan. 1998. *Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell* **94**: 491-501.

Lipoldova, M., A.W. Boylston, H. Yssel and M.J. Owen. 1989. *T-cell receptor V beta 5 usage defines reactivity to a human T-cell receptor monoclonal antibody. Immunogenetics* **30**: 162-8

Liu, R., W.A. Paxton, S. Choe, D. Ceradini, S.R. Martin, R. Horuk, M.E. MacDonald, H. Stuhlmann, R.A. Koup, and N.R. Landau. 1996a. *Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell* **86**: 367-77.

Liu, X., C.N. Kim, J. Yang, R. Jemmerson, and X. Wang. 1996b. *Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell* **86**: 147-57.

Liu, X., H. Zou, C. Slaughter, and X. Wang. 1997. *DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell* **89**: 175-84.

Loetscher, H., Y.C. Pan, H.W. Lahm, R. Gentz, M. Brockhaus, H. Tabuchi, and W. Lesslauer. 1990. *Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell* **61**: 351-9.

Lu, Z., J.F. Berson, Y. Chen, J.D. Turner, T. Zhang, M. Sharron, M.H. Jenks, Z. Wang, J. Kim, J. Rucker, J.A. Hoxie, S.C. Peiper, and R.W. Doms. 1997. *Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. Proc Natl Acad Sci U S A* **94**: 6426-31.

Luo, X., I. Budihardjo, H. Zou, C. Slaughter, and X. Wang. 1998. *Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell* **94**: 481-90.

Maghazachi, A.A. 1997. *Role of the heterotrimeric G proteins in stromal-derived factor-1alpha- induced natural killer cell chemotaxis and calcium mobilization [published erratum appears in Biochem Biophys Res Commun 1997 Aug 28;237(3):759]. Biochem Biophys Res Commun* **236**: 270-4.

Marsters, S.A., J.P. Sheridan, C.J. Donahue, R.M. Pitti, C.L. Gray, A.D. Goddard, K.D. Bauer, and A. Ashkenazi. 1996. *Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NF-kappa B. Curr Biol* **6**: 1669-76.

Marsters, S.A., J.P. Sheridan, R.M. Pitti, J. Brush, A. Goddard, and A. Ashkenazi. 1998. *Identification of a ligand for the death-domain-containing receptor Apo3. Curr Biol* **8**: 525-8.

Martinson, J.J., N.H. Chapman, D.C. Rees, Y.T. Liu, and J.B. Clegg. 1997. *Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet* **16**: 100-3.

Mashima, T., M. Naito, N. Fujita, K. Noguchi, and T. Tsuruo. 1995. *Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. Biochem Biophys Res Commun* **217**: 1185-92.

McCarthy, N.J., M.K. Whyte, C.S. Gilbert, and G.I. Evan. 1997. *Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. J Cell Biol* **136**: 215-27.

McDermott, D.H., P.A. Zimmerman, F. Guignard, C.A. Kleeberger, S.F. Leitman, and P.M. Murphy. 1998. *CCR5 promoter polymorphism and HIV-1 disease progression. Multicenter AIDS Cohort Study (MACS). Lancet* **352**: 866-70.

McMichael, A.J. and R.E. Phillips. 1997. *Escape of human immunodeficiency virus from immune control. Annu Rev Immunol* **15**: 271-96.

Mellors, J.W., C.R. Rinaldo, Jr., P. Gupta, R.M. White, J.A. Todd, and L.A. Kingsley. 1996. *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science* **272**: 1167-70.

Meyaard, L., S.A. Otto, R.R. Jonker, M.J. Mijnster, R.P. Keet, and F. Miedema. 1992. *Programmed death of T cells in HIV-1 infection. Science* **257**: 217-9.

Miethke, T., R. Vabulas, R. Bittlingmaier, K. Heeg, and H. Wagner. 1996. *Mechanisms of peripheral T cell deletion: anergized T cells are Fas resistant but undergo proliferation-associated apoptosis. Eur J Immunol* **26**: 1459-67.

Miller, R. and Siegmund, D. 1982. *Maximally selected chi-square statistics. Biometrics* **38**: 1011-1016

Miura, Y., N. Misawa, N. Maeda, Y. Inagaki, Y. Tanaka, M. Ito, N. Kayagaki, N. Yamamoto, H. Yagita, H. Mizusawa, and Y. Koyanagi. 2001. *Critical contribution of tumor necrosis factor-related apoptosis- inducing ligand (TRAIL) to apoptosis of human CD4+ T cells in HIV-1- infected hu-PBL-NOD-SCID mice. J Exp Med* **193**: 651-60.

Mosmann, T. 1983. *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods* **65**: 55-63.

Moutouh, L., J. Estaquier, D.D. Richman, and J. Corbeil. 1998. *Molecular and cellular analysis of human immunodeficiency virus-induced apoptosis in lymphoblastoid T-cell-line-expressing wild-type and mutated CD4 receptors. J Virol* **72**: 8061-72.

Muller, C., S. Kukel, and R. Bauer. 1994. *Antibodies against CD4+ lymphocytes in plasma of HIV-infected patients are related to CD4 cell depletion in vivo. Immunol Lett* **41**: 163-7.

Muro-Cacho, C.A., G. Pantaleo, and A.S. Fauci. 1995. *Analysis of apoptosis in lymph nodes of HIV-infected persons. Intensity of apoptosis correlates with the general state of activation of the lymphoid tissue and not with stage of disease or viral burden. J Immunol* **154**: 5555-66.

Murphy, M.A., R.G. Schnall, D.J. Venter, L. Barnett, I. Bertoncello, C.B. Thien, W.Y. Langdon, and D.D. Bowtell. 1998. *Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl- deficient mice. Mol Cell Biol* **18**: 4872-82.

Muzio, M., A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, M. Mann, P.H. Krammer, M.E. Peter, and V.M. Dixit. 1996. *FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell* **85**: 817-27.

Nagata, S. 1997. *Apoptosis by death factor. Cell* **88**: 355-65.

Nagata, S. 2000. *Apoptotic DNA fragmentation. Exp Cell Res* **256**: 12-8.

Nakajima, H., P. Golstein, and P.A. Henkart. 1995. *The target cell nucleus is not required for cell-mediated granzyme- or Fas-based cytotoxicity. J Exp Med* **181**: 1905-9.

Neumann, E., M. Schaefer-Ridder, Y. Wang, and P.H. Hofschneider. 1982. *Gene transfer into mouse lyoma cells by electroporation in high electric fields. Embo J* **1**: 841-5.

Nicoletti, I., G. Migliorati, M.C. Pagliacci, F. Grignani, and C. Riccardi. 1991. *A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods* **139**: 271-9.

Nicotera, P., M. Leist, E. Fava, L. Berliocchi, and C. Volbracht. 2000. *Energy requirement for caspase activation and neuronal cell death. Brain Pathol* **10**: 276-82.

Nilsson, N., M. Wallen-Ohman, M. Ohlin, and C.A. Borrebaeck. 1997. *Altered gene expression associated with apoptosis in a pre-B-leukemic cell line following cross-linking of MHC class I. Exp Cell Res* **231**: 190-7.

Noraz, N., J. Gozlan, J. Corbeil, T. Brunner, and S.A. Spector. 1997. *HIV-induced apoptosis of activated primary CD4+ T lymphocytes is not mediated by Fas-Fas ligand. Aids* **11**: 1671-80.

O'Brien, T.R., C. Winkler, M. Dean, J.A. Nelson, M. Carrington, N.L. Michael, and G.C. White, 2nd. 1997. *HIV-1 infection in a man homozygous for CCR5 delta 32. Lancet* **349**: 1219.

Oh, S.K., W.W. Cruikshank, J. Raina, G.C. Blanchard, W.H. Adler, J. Walker, and H. Kornfeld. 1992. *Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. J Acquir Immune Defic Syndr* **5**: 251-6.

Ohnimus, H., M. Heinkelein, and C. Jassoy. 1997. *Apoptotic cell death upon contact of CD4+ T lymphocytes with HIV glycoprotein-expressing cells is mediated by caspases but bypasses CD95 (Fas/Apo-1) and TNF receptor 1. J Immunol* **159**: 5246-52.

Okada, H., R. Takei, and M. Tashiro. 1997. *HIV-1 Nef protein-induced apoptotic cytolysis of a broad spectrum of uninfected human blood cells independently of CD95(Fas). FEBS Lett* **414**: 603-6.

Okuno, S., S. Shimizu, T. Ito, M. Nomura, E. Hamada, Y. Tsujimoto, and H. Matsuda. 1998. *Bcl-2 prevents caspase-independent cell death. J Biol Chem* **273**: 34272-7.

Oyaizu, N., T.W. McCloskey, M. Coronesi, N. Chirmule, V.S. Kalyanaraman, and S. Pahwa. 1993. *Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood* **82**: 3392-400.

Oyaizu, N. and S. Pahwa. 1995. *Role of apoptosis in HIV disease pathogenesis. J Clin Immunol* **15**: 217-31.

Pan, G., J. Ni, Y.F. Wei, G. Yu, R. Gentz, and V.M. Dixit. 1997a. *An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science* **277**: 815-8.

Pan, G., K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V.M. Dixit. 1997b. *The receptor for the cytotoxic ligand TRAIL. Science* **276**: 111-3.

Pantaleo, G., J.F. Demarest, H. Soudeyns, C. Graziosi, F. Denis, J.W. Adelsberger, P. Borrow, M.S. Saag, G.M. Shaw, R.P. Sekaly, and et al. 1994a. *Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. Nature* **370**: 463-7.

Pantaleo, G., C. Graziosi, J.F. Demarest, O.J. Cohen, M. Vaccarezza, K. Gantt, C. Muro-Cacho, and A.S. Fauci. 1994b. *Role of lymphoid organs in the pathogenesis of human immunodeficiency virus (HIV) infection. Immunol Rev* **140**: 105-30.

Penn, M.L., J.C. Grivel, B. Schramm, M.A. Goldsmith, and L. Margolis. 1999. *CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV- 1-infected human lymphoid tissue. Proc Natl Acad Sci U S A* **96**: 663-8.

Peppel, K., D. Crawford, and B. Beutler. 1991. *A tumor necrosis factor (TNF) receptor-IgG heavy chain chimeric protein as a bivalent antagonist of TNF activity. J Exp Med* **174**: 1483-9.

Pettersen, R.D., K. Hestdal, M.K. Olafsen, S.O. Lie, and F.P. Lindberg. 1999. *CD47 signals T cell death. J Immunol* **162**: 7031-40.

Pezzutto, A., B. Dorken, P.S. Rabinovitch, J.A. Ledbetter, G. Moldenhauer, and E.A. Clark. 1987. *CD19 monoclonal antibody HD37 inhibits anti-immunoglobulin-induced B cell activation and proliferation. J Immunol* **138**: 2793-9.

Phenix, B.N., Angel, J.B., Mandy, F., Kravcik, S., Parato, K., Chambers, K.A., Gallicano, K., Hawley-Foss, N., Cassol, S., Cameron, D.W., and Badley, A.D. 2000. *Decreased HIV-associated T cell apoptosis by HIV protease inhibitors. AIDS Res Hum Retroviruses* **16**: 559-67.

Pierson, T., J. McArthur, and R.F. Siliciano. 2000. *Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. Annu Rev Immunol* **18**: 665-708.

Pitcher, C., S. Honing, A. Fingerhut, K. Bowers, and M. Marsh. 1999. *Cluster of differentiation antigen 4 (CD4) endocytosis and adaptor complex binding require activation of the CD4 endocytosis signal by serine phosphorylation. Mol Biol Cell* **10**: 677-91.

Popik, W. and P.M. Pitha. 1998. *Early activation of mitogen-activated protein kinase kinase, extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, and*

c-Jun N-terminal kinase in response to binding of simian immunodeficiency virus to Jurkat T cells expressing CCR5 receptor. Virology **252**: 210-7.

Quignon, F., F. De Bels, M. Koken, J. Feunteun, J.C. Ameisen, and H. de The. 1998. *PML induces a novel caspase-independent death process. Nat Genet* **20**: 259-65.

Rana, S., G. Besson, D.G. Cook, J. Rucker, R.J. Smyth, Y. Yi, J.D. Turner, H.H. Guo, J.G. Du, S.C. Peiper, E. Lavi, M. Samson, F. Libert, C. Liesnard, G. Vassart, R.W. Doms, M. Parmentier, and R.G. Collman. 1997. *Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the delta ccr5 mutation. J Virol* **71**: 3219-27.

Rao, L., D. Perez, and E. White. 1996. *Lamin proteolysis facilitates nuclear events during apoptosis. J Cell Biol* **135**: 1441-55.

Raport, C.J., J. Gosling, V.L. Schweickart, P.W. Gray, and I.F. Charo. 1996. *Molecular cloning and functional characterization of a novel human CC chemokine receptor (CCR5) for RANTES, MIP-1beta, and MIP-1alpha. J Biol Chem* **271**: 17161-6.

Rouleau, M., A. Bernard, O. Lantz, J.P. Vernant, B. Charpentier, and A. Senik. 1993. *Apoptosis of activated CD8+/CD57+ T cells is induced by some combinations of anti-CD2 mAb. J Immunol* **151**: 3547-56.

Russell, J.H., C.L. White, D.Y. Loh, and P. Meleedy-Rey. 1991. *Receptor-stimulated death pathway is opened by antigen in mature T cells. Proc Natl Acad Sci U S A* **88**: 2151-5.

Sarin, A., M.L. Wu, and P.A. Henkart. 1996. *Different interleukin-1 beta converting enzyme (ICE) family protease requirements for the apoptotic death of T lymphocytes triggered by diverse stimuli. J Exp Med* **184**: 2445-50.

Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, and M.E. Peter. 1998. *Two CD95 (APO-1/Fas) signaling pathways. Embo J* **17**: 1675-87.

Scaffidi, C., J.P. Medema, P.H. Krammer, and M.E. Peter. 1997. *FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. J Biol Chem* **272**: 26953-8.

Scarlatti, G., E. Tresoldi, A. Bjorndal, R. Fredriksson, C. Colognesi, H.K. Deng, M.S. Malnati, A. Plebani, A.G. Siccardi, D.R. Littman, E.M. Fenyo, and P. Lusso. 1997. *In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat Med* **3**: 1259-65.

Schall, T.J., M. Lewis, K.J. Koller, A. Lee, G.C. Rice, G.H. Wong, T. Gatanaga, G.A. Granger, R. Lentz, H. Raab, and et al. 1990. *Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell* **61**: 361-70.

Schlapbach, R. and A. Fontana. 1997. *Differential activity of bcl-2 and ICE enzyme family protease inhibitors on Fas and puromycin-induced apoptosis of glioma cells. Biochim Biophys Acta* **1359**: 174-80.

Schmidtke, G., Holzhutter, H.G., Bogyo, M., Kairies, N., Groll, M., de Giuli, R., Emch, S., and Groettrup, M. 1999. *How an inhibitor of the HIV-I protease modulates proteasome activity. J BiolChem* **274**: 35734-40.

Schneider, P., J.L. Bodmer, M. Thome, K. Hofmann, N. Holler, and J. Tschopp. 1997. *Characterization of two receptors for TRAIL. FEBS Lett* **416**: 329-34.

Schols, D., S. Struyf, J. Van Damme, J.A. Este, G. Henson, and E. De Clercq. 1997. *Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J Exp Med* **186**: 1383-8.

Schotte, P., W. Van Criekinge, M. Van de Craen, G. Van Loo, M. Desmedt, J. Grooten, M. Cornelissen, L. De Ridder, J. Vandekerckhove, W. Fiers, P. Vandenabeele, and R. Beyaert. 1998. *Cathepsin B-mediated activation of the proinflammatory caspase-11. Biochem Biophys Res Commun* **251**: 379-87.

Schramm, B., M.L. Penn, R.F. Speck, S.Y. Chan, E. De Clercq, D. Schols, R.I. Connor, and M.A. Goldsmith. 2000. *Viral entry through CXCR4 is a pathogenic factor and therapeutic target in human immunodeficiency virus type 1 disease. J Virol* **74**: 184-92.

Schuitemaker, H., M. Koot, N.A. Kootstra, M.W. Dercksen, R.E. de Goede, R.P. van Steenwijk, J.M. Lange, J.K. Schattenkerk, F. Miedema, and M. Tersmette. 1992. *Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J Virol* **66**: 1354-60.

Schulze-Osthoff, K., H. Walczak, W. Droge, and P.H. Krammer. 1994. *Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol* **127**: 15-20.

Schwartz, D.C. and C.R. Cantor. 1984. *Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell* **37**: 67-75.

Screaton, G.R., J. Mongkolsapaya, X.N. Xu, A.E. Cowper, A.J. McMichael, and J.I. Bell. 1997. *TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. Curr Biol* **7**: 693-6.

Sheridan, J.P., S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, A.D. Goddard, P. Godowski, and A. Ashkenazi. 1997. *Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science* **277**: 818-21.

Simmonds, P., P. Balfe, J.F. Peutherer, C.A. Ludlam, J.O. Bishop, and A.J. Brown. 1990. *Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J Virol* **64**: 864-72.

Slee, E.A., M.T. Harte, R.M. Kluck, B.B. Wolf, C.A. Casiano, D.D. Newmeyer, H.G. Wang, J.C. Reed, D.W. Nicholson, E.S. Alnemri, D.R. Green, and S.J. Martin. 1999. *Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9- dependent manner. J Cell Biol* **144**: 281-92.

Sloand, E.M., Maciejewski, J., Kumar, P., Kim, S., Chaudhuri, A., and Young, N. 2000. *Protease inhibitors stimulate hematopoiesis and decrease apoptosis and ICE expression in CD34(+) cells. Blood* **96**: 2735-9

Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. *The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell* **76**: 959-62.

Smith, M.W., M. Dean, M. Carrington, C. Winkler, G.A. Huttley, D.A. Lomb, J.J. Goedert, T.R. O'Brien, L.P. Jacobson, R. Kaslow, S. Buchbinder, E. Vittinghoff, D. Vlahov, K. Hoots, M.W. Hilgartner, and S.J. O'Brien. 1997. *Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC), ALIVE Study. Science* **277**: 959-65.

Song, Q., S.P. Lees-Miller, S. Kumar, Z. Zhang, D.W. Chan, G.C. Smith, S.P. Jackson, E.S. Alnemri, G. Litwack, K.K. Khanna, and M.F. Lavin. 1996. *DNA-dependent protein kinase catalytic subunit: a target for an ICE- like protease in apoptosis. Embo J* **15**: 3238-46.

Sperandio, S., I. de Belle, and D.E. Bredesen. 2000. *An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A* **97**: 14376-81.

Srinivasula, S.M., M. Ahmad, T. Fernandes-Alnemri, and E.S. Alnemri. 1998. *Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell* **1**: 949-57.

Stewart, S.A., B. Poon, J.B. Jowett, and I.S. Chen. 1997. *Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. J Virol* **71**: 5579-92.

Susin, S.A., H.K. Lorenzo, N. Zamzami, I. Marzo, B.E. Snow, G.M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D.R. Goodlett, R. Aebersold, D.P. Siderovski, J.M. Penninger, and G. Kroemer. 1999. *Molecular characterization of mitochondrial apoptosis-inducing factor. Nature* **397**: 441-6.

Tanke, H.J., P.W. van der Linden, and J. Langerak. 1982. *Alternative fluorochromes to ethidium bromide for automated read out of cytotoxicity tests. J Immunol Methods* **52**: 91-6.

Tartaglia, L.A., T.M. Ayres, G.H. Wong, and D.V. Goeddel. 1993. *A novel domain within the 55 kd TNF receptor signals cell death. Cell* **74**: 845-53.

Tewari, M., D.R. Beidler, and V.M. Dixit. 1995. *CrmA-inhibitable cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein during Fas- and tumor necrosis factor- induced apoptosis. J Biol Chem* **270**: 18738-41.

Theodorou, I., L. Meyer, M. Magierowska, C. Katlama, and C. Rouzioux. 1997. *HIV-1 infection in an individual homozygous for CCR5 delta 32. Seroco Study Group. Lancet* **349**: 1219-20.

Thornberry, N.A., T.A. Rano, E.P. Peterson, D.M. Rasper, T. Timkey, M. Garcia-Calvo, V.M. Houtzager, P.A. Nordstrom, S. Roy, J.P. Vaillancourt, K.T. Chapman, and D.W. Nicholson. 1997. *A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem* **272**: 17907-11.

Towbin, H., T. Staehelin, and J. Gordon. 1979. *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A* **76**: 4350-4.

Trauth, B.C., C. Klas, A.M. Peters, S. Matzku, P. Moller, W. Falk, K.M. Debatin, and P.H. Krammer. 1989. *Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science* **245**: 301-5.

Truman, J.P., M.L. Ericson, C.J. Choqueux-Seebold, D.J. Charron, and N.A. Mooney. 1994. *Lymphocyte programmed cell death is mediated via HLA class II DR. Int Immunol* **6**: 887-96.

Ugolini, S., M. Moulard, I. Mondor, N. Barois, D. Demandolx, J. Hoxie, A. Brelot, M. Alizon, J. Davoust, and Q.J. Sattentau. 1997. *HIV-1 gp120 induces an association between CD4 and the chemokine receptor CXCR4. J Immunol* **159**: 3000-8.

Ulivieri, C., S. Pacini, S. Bartalini, S. Valensin, J.L. Telford, and C.T. Baldari. 1999. *Obligatory cross-talk with the tyrosine kinases assembled with the TCR/CD3 complex in CD4 signal transduction. Eur J Immunol* **29**: 2625-35.

van Leeuwen, J.E., P.K. Paik, and L.E. Samelson. 1999. *The oncogenic 70Z Cbl mutation blocks the phosphotyrosine binding domain-dependent negative regulation of ZAP-70 by c-Cbl in Jurkat T cells. Mol Cell Biol* **19**: 6652-64.

Verrier, F.C., P. Charneau, R. Altmeyer, S. Laurent, A.M. Borman, and M. Girard. 1997. *Antibodies to several conformation-dependent epitopes of gp120/gp41 inhibit CCR-5-dependent cell-to-cell fusion mediated by the native envelope glycoprotein of a primary macrophage-tropic HIV-1 isolate. Proc Natl Acad Sci U S A* **94**: 9326-31.

Vila-Coro, A.J., J.M. Rodriguez-Frade, A. Martin De Ana, M.C. Moreno-Ortiz, A.C. Martinez, and M. Mellado. 1999. *The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. Faseb J* **13**: 1699-710.

Vlahakis, S.R., A. Algeciras-Schimnich, G. Bou, C.J. Heppelmann, A. Villasis-Keever, R.C. Collman, and C.V. Paya. 2001. *Chemokine-receptor activation by env determines*

the mechanism of death in HIV-infected and uninfected T lymphocytes. J Clin Invest **107**: 207-15.

Vogt, C. (1842) Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkroete (Alytes obstetricans).

Walczak, H., M.A. Degli-Esposti, R.S. Johnson, P.J. Smolak, J.Y. Waugh, N. Boiani, M.S. Timour, M.J. Gerhart, K.A. Schooley, C.A. Smith, R.G. Goodwin, and C.T. Rauch. 1997. *TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. Embo J* **16**: 5386-97.

Walker, P.R., L. Kokileva, J. LeBlanc, and M. Sikorska. 1993. *Detection of the initial stages of DNA fragmentation in apoptosis. Biotechniques* **15**: 1032-40.

Wallen-Ohman, M., J.W. Larrick, R. Carlsson, and C.A. Borrebaeck. 1997. *Ligation of MHC class I induces apoptosis in human pre-B cell lines, in promyelocytic cell lines and in CD40-stimulated mature B cells. Int Immunol* **9**: 599-606.

Waterhouse, N., S. Kumar, Q. Song, P. Strike, L. Sparrow, G. Dreyfuss, E.S. Alnemri, G. Litwack, M. Lavin, and D. Watters. 1996. *Heteronuclear ribonucleoproteins C1 and C2, components of the spliceosome, are specific targets of interleukin 1beta-converting enzyme-like proteases in apoptosis. J Biol Chem* **271**: 29335-41.

Waterhouse, N.J., D.M. Finucane, D.R. Green, J.S. Elce, S. Kumar, E.S. Alnemri, G. Litwack, K. Khanna, M.F. Lavin, and D.J. Watters. 1998. *Calpain activation is upstream of caspases in radiation-induced apoptosis. Cell Death Differ* **5**: 1051-61.

Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, and et al. 1995. *Viral dynamics in human immunodeficiency virus type 1 infection. Nature* **373**: 117-22.

Weichold, F.F., Bryant, J.L., Pati, S., Barabitskaya, O., Gallo, R.C., and Reitz, M.S. 1999. *HIV-1 protease inhibitor ritonavir modulates susceptibility to apoptosis of uninfected T cells. J Hum Virol* **2**: 261-9.

Weiss, A. and D.R. Littman. 1994. *Signal transduction by lymphocyte antigen receptors. Cell* **76**: 263-74.

Wesselborg, S., U. Prufer, M. Wild, B. Schraven, S.C. Meuer, and D. Kabelitz. 1993. *Triggering via the alternative CD2 pathway induces apoptosis in activated human T lymphocytes. Eur J Immunol* **23**: 2707-10.

Westendorp, M.O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K.M. Debatin, and P.H. Krammer. 1995a. *Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature* **375**: 497-500.

Westendorp, M.O., V.A. Shatrov, K. Schulze-Osthoff, R. Frank, M. Kraft, M. Los, P.H. Krammer, W. Droge, and V. Lehmann. 1995b. *HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. Embo J* **14**: 546-54.

Wiley, S.R., K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, and et al. 1995. *Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity* **3**: 673-82.

Williams, M.S. and P.A. Henkart. 1994. *Apoptotic cell death induced by intracellular proteolysis. J Immunol* **153**: 4247-55.

Winkler, C., W. Modi, M.W. Smith, G.W. Nelson, X. Wu, M. Carrington, M. Dean, T. Honjo, K. Tashiro, D. Yabe, S. Buchbinder, E. Vittinghoff, J.J. Goedert, T.R. O'Brien, L.P. Jacobson, R. Detels, S. Donfield, A. Willoughby, E. Gomperts, D. Vlahov, J. Phair, and S.J. O'Brien. 1998. *Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. ALIVE Study, Hemophilia Growth and Development Study (HGDS), Multicenter AIDS Cohort Study (MACS), Multicenter Hemophilia Cohort Study (MHCS), San Francisco City Cohort (SFCC) [see comments]. Science* **279**: 389-93.

Woodle, E.S., D.M. Smith, J.A. Bluestone, W.M. Kirkman, 3rd, D.R. Green, and E.W. Skowronski. 1997. *Anti-human class I MHC antibodies induce apoptosis by a pathway that is distinct from the Fas antigen-mediated pathway. J Immunol* **158**: 2156-64.

Wu, L., G. LaRosa, N. Kassam, C.J. Gordon, H. Heath, N. Ruffing, H. Chen, J. Humblias, M. Samson, M. Parmentier, J.P. Moore, and C.R. Mackay. 1997. *Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J Exp Med* **186**: 1373-81.

Wyllie, A.H. 1980. *Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature* **284**: 555-6.

Xiang, J., D.T. Chao, and S.J. Korsmeyer. 1996. *BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. Proc Natl Acad Sci U S A* **93**: 14559-63.

Xiao, L., D.L. Rudolph, S.M. Owen, T.J. Spira, and R.B. Lal. 1998. *Adaptation to promiscuous usage of CC and CXC-chemokine coreceptors in vivo correlates with HIV-1 disease progression. Aids* **12**: F137-43.

Yi, Y., S.N. Isaacs, D.A. Williams, I. Frank, D. Schols, E. De Clercq, D.L. Kolson, and R.G. Collman. 1999. *Role of CXCR4 in cell-cell fusion and infection of monocyte-derived macrophages by primary human immunodeficiency virus type 1 (HIV-1) strains: two distinct mechanisms of HIV-1 dual tropism. J Virol* **73**: 7117-25.

Yonehara, S., A. Ishii, and M. Yonehara. 1989. *A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med* **169**: 1747-56.

Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J.L. Vayssiere, P.X. Petit, and G. Kroemer. 1995. *Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med* **181**: 1661-72.

Zanke, B.W., C. Lee, S. Arab, and I.F. Tannock. 1998. *Death of tumor cells after intracellular acidification is dependent on stress-activated protein kinases (SAPK/JNK) pathway activation and cannot be inhibited by Bcl-2 expression or interleukin 1beta-converting enzyme inhibition. Cancer Res* **58**: 2801-8.

Zeitlmann, L., P. Sirim, E. Kremmer, and W. Kolanus. 2001. *Cloning of ACP33 as a novel intracellular ligand of CD4. J Biol Chem* **276**: 9123-32.

Zhang, M., X. Li, X. Pang, L. Ding, O. Wood, K. Clouse, I. Hewlett, and A.I. Dayton. 2001. *Identification of a potential HIV-induced source of bystander-mediated apoptosis in T cells: upregulation of trail in primary human macrophages by HIV-1 tat. J Biomed Sci* **8**: 290-6.

Zheng, J., M.R. Thylin, A. Ghorpade, H. Xiong, Y. Persidsky, R. Cotter, D. Niemann, M. Che, Y.C. Zeng, H.A. Gelbard, R.B. Shepard, J.M. Swartz, and H.E. Gendelman. 1999. *Intracellular CXCR4 signaling, neuronal apoptosis and neuropathogenic mechanisms of HIV-1-associated dementia. J Neuroimmunol* **98**: 185-200.

Zou, H., W.J. Henzel, X. Liu, A. Lutschg, and X. Wang. 1997. *Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3 [see comments]. Cell* **90**: 405-13.

Zou, H., Y. Li, X. Liu, and X. Wang. 1999. *An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem* **274**: 11549-56.

VI Abbreviations

#	number
α	anti-
Ψm	mitochondrial transmembrane potential
ΔΨm	mitochondrial transmembrane potential reduction
λ	wavelength
µg	microgramm
µl	microlitre
µM	micromolar
Ab	antibody
AICD	activation induced cell death
AIDS	acquired immunodeficiency syndrome
AIF	apoptosis inducing faktor
Act D	actinomycin D
APS	ammonium Peroxide sulfate
Ag	antigen
ATP	adenosine triphosphate
Bcl-2	B cell lymphoma
BD.fmk	t-butoxy carbonyl-Asp.fluoromethylketone
bp	base pair
BSA	bovine serum albumin
CD95L	CD95 ligand
CCR	C-C-chemokine receptor
CD	cluster of differentiation
cDNA	complementary deoxyribonnucleic acid
Ci	Curie

CIAP	calf intestinal alkaline phosphatase
CrmA	cytokine response modifier A
CTL	cytotoxic T cell
CXCR	C-X-C chemokine receptor
Cyt-C	cytochrome c
Da	Dalton
DD	death domain
DED	death effector domain
DFF	DNA fragmentation factor
DISC	death inducing signaling complex
$DiOC_6(3)$	dihexyloxacarbocyanine iodide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
EDTA	ethyl diamine tetra-acetate
env	envelope
ERK	extracellular signal related kinase
FACS	fluorescence-activated cell sorter
FADD	Fas-associated death domain protein
FCS	fetal calf serume
FITC	fluorescein isothiocyanate
FL	fluorescence
FLICE	FADD-like ICE
fmk	fluoromethyl ketone
FSC	forward scatter
gp	glycoprotein
h	hour
HIV-1	human immundeficiency virus 1
HPBALL	human peripheral blood acute lymphatic leukemia
HRPO	horse raddish-peroxidase
ICE	Interleukin-1β–converting enzyme
IFN	Interferon

Ig	Immunglobulin
IL	Interleukin
kDa	kilodalton
kb	kilobase
lck	lymphocyte specific protein tyrosine kinase
LT	lymphotoxin
LZ	Leucine-Zipper
M	molar
MAPK	mitogen-aktiviated protein kinase
MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MIP	macrophage inflammatory protein
min.	minute
ml	mililitre
mM	milimolar
MMP	mitochondrial membrane permeabilisation
MORT1	mediator of receptor-induced toxicity 1
M-tropic	macrophage tropic
MW	molecular weight
NK cells	natural killer cells
NO	nitric oxide
NSI	non syncytium inducing
OD	optical density
PARP	poly-(ADP-ribose)-polymerase
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase-chain reaction
PE	phycoerythrin
PHA	phytohemagglutinin
PKC	Proteinkinase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonylfluoride

PT	permeability-transition
RANTES	regulated upon activation, T-expressed and secreted
rpm	rounds per minute
RT	room temperature
SDS-PAGE	Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese
sec	second
SDF-1	stromal cell-derived factor 1
SDS	sodium dodecyl sulfate
src	sarcoma virus protein kinase
SSC	side scatter
t	time
TBE	Tris-borate-EDTA
TCR	T-cell-receptor
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor-receptor
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAMP	TNF receptor-related apoptosis mediating protein
T-tropic	T cell tropic
UV	ultraviolet
v/v	volume per volume
v/w	volume per weight
zVAD.fmk	Benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluorometyl ketone